U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371 AND 37 CFR 1.491**	ATTORNEY DOCKET NO. 214616 U.S. APPLICATION NO. Unassigned 10/018396

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/16628	16 June 2000	18 June 1999

TITLE OF INVENTION

EXTRACELLULAR cAMP-DEPENDENT PROTEIN KINASE IN DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER

APPLICANT(S) FOR DO/EO/US

Cho-Chung

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.

3. ☒ This is an express request to begin national examination procedures (35 USC 371(f)).

4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).

5. ☒ A copy of the International Application as filed (35 USC 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ An English language translation of the International Application as filed (35 USC 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 USC 371(c)(4)).

10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

11. Nucleotide and/or Amino Acid Sequence Submission
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper Copy
   c. ☐ Statement verifying identity of above copies

Items 12 to 19 below concern other document(s) or information included:

12. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
   ☐ Form PTO-1449
   ☐ Copies of Listed Documents

13. ☐ An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. ☐ A FIRST preliminary amendment.
   ☐ A SECOND or SUBSEQUENT preliminary amendment.

15. ☐ A substitute specification.

16. ☐ A change of power of attorney and/or address letter.

17. ☒ Application Data Sheet Under 37 CFR 1.76

18. ☒ Return Receipt Postcard

19. ☐ Other items or information:

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NO.
Unassigned 10/018396	PCT/US00/16628	214616

20. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,040.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... $ 890.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO, but international search fee (37 CFR 1.445(a)(2))paid to USPTO ..... $ 740.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... $ 710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) to (4) ..... $ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT=	$890.00	
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date	$	

CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	20	-20=	0	x $ 18.00	$	
Independent Claims	5	- 3 =	2	x $ 84.00	$168.00	
☐ Multiple Dependent Claim(s) (if applicable)				+$280.00	$	

TOTAL OF ABOVE CALCULATIONS=	$	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.	$	
SUBTOTAL=	$1,058.00	
Processing fee of **$130.00** for furnishing English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date.	$	
TOTAL NATIONAL FEE=	$1,058.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. **$40.00** per property +	$	
TOTAL FEE ENCLOSED=	$1,058.00	
	Amount to be: refunded	$
	charged:	$

a. ☒ A check in the amount of $1,058.00 to cover the above fee is enclosed.

b. ☐ Please charge Deposit Account No. 12-1216 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1216. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Customer Number: 23460

23460

PATENT TRADEMARK OFFICE

Carol Larcher

Carol Larcher, Registration No. 35,243
One of the Attorneys for Applicant(s)

Date: December 13, 2001

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NO.
Unassigned 10/018396	PCT/US00/16628	214616

CERTIFICATION UNDER 37 CFR 1.10

"Express Mail" Label Number: EL 841012518 US

Date of Deposit: December 13, 2001

I hereby certify that this express request to begin national examination procedures under 35 USC 371(f) of the International Patent Application referenced above, including all of the items listed thereon as enclosures, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Commissioner for Patents, Attention: DO/EO/US, Washington, D.C. 20231.

Virginia Schefke
Printed Name of Person Signing:

Virginia Schefke
Signature

EXTRACELLULAR CAMP-DEPENDENT PROTEIN KINASE IN DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER

TECHNICAL FIELD OF THE INVENTION

The present invention relates to methods of diagnosing and prognosticating cancer comprising assaying extracellular cAMP-dependent protein kinase (ECPKA). The present invention also relates to methods of treating cancer comprising inhibiting the expression of extracellular cAMP-dependent protein kinase or the type II isozyme thereof.

BACKGROUND OF THE INVENTION

The extrusion of cAMP from intact animal cells was discovered by Davoren and Sutherland (*J. Biol. Chem.* 238: 3009-3015 (1963)) on catecholamine-stimulated pigeon erythrocytes. Since that time, the egress of cAMP from a variety of tissues, cultured cells, and lower forms of organisms, including the slime mold *Dictyostelium discoideum* and bacteria, has been described (Barber and Butcher, In *Advances in Cyclic Nucleotides Research*, Greengard et al., eds., pp. 119-138, Raven Press, NY (1983)).

The physiological role of extracellular cAMP is partly known for some lower forms of organisms. In *D. discoideum,* chemotaxis and cell differentiation appear to be regulated by extracellular cAMP pulses (Darmon et al., *PNAS USA* 72: 3163-3166 (1975)). It was shown that chemotaxis and cAMP signaling are mediated by special cAMP-binding receptor proteins located on the surface of the cells (Bonner, In *The Development of Dictyostelium discoideum*, Lommis, ed., pp. 1-33, Academic Press, NY (1982); and Theibert et al., *J. Biol. Chem.* 250: 12318-12381 (1983)).

Functions of extracellular cAMP, especially for animal cells, still remain obscure. Under some conditions, the cumulative extracellular quantity of the cAMP appears to reflect the influence of various agents on cAMP generation and cell damage (Broadus et al., *Ann. N.Y. Acad. Sci.* 185: 50-66 (1971)). However, cAMP efflux from the cells cannot be explained only by destruction of cells. It has been shown that, in avian erythrocytes and cultured mammalian cells, cAMP is released by

an energy-dependent mechanism that has some properties of active transport (Davoren and Sutherland (1963), *supra*; Rindler et al., *J. Biol. Chem.* 253: 5431-5436 (1978); and Barber and Butcher (1983), *supra*). A number of pharmacological and hormonal agents inhibit this process (Rindler et al. (1978), *supra*; and Heasley and Brunton, *J. Biol. Chem.* 260: 11514-11519 (1985)). The action of some of them is not related to alterations of adenylate cyclase activity or the level of cellular ATP (Rindler et al. (1978), *supra*).

Because the effects of cAMP in mammalian cells are largely mediated by cAMP-dependent protein kinase (PKA) (Krebs and Beavo, *Ann. Rev. Biochem.* 48: 923-939 (1979)), it is conceivable that the effluxed cAMP from the cell may have some physiological significance in regulating cell surface-located protein kinase which is cAMP-dependent, namely, ecto-PKA. Furthermore, the cell surface-located PKA may play an important role in functions such as cell motility, cell adhesion, cell-cell interaction, or cell reception and transduction of external signals.

The presence of PKA on the external surface of LS-174T human colon carcinoma cells has recently been discovered (Kondrashin et al., *Biochemistry* 38: 172-179 (1999)). This ecto-PKA is immunologically related to the intracellular soluble PKA. The ecto-PKA is stimulated by cAMP in phosphorylating a synthetic peptide substrate of PKA, kemptide, and is specifically inhibited by PKA inhibitory protein, PKI (Walsh-Krebs inhibitor). The source of cAMP for activating the ecto-PKA comes from the intracellular source upon its secretion after forskolin treatment. Probenecid, which inhibits the secretion of cAMP, blocks the forskolin-mediated activation of ecto-PKA.

In mammalian cells, there are two types of PKA, type I (PKA-I) and type II (PKA-II), which share a common C subunit but contain distinct R subunits, RI and RII, respectively (Beebe and Corbin, In *The Enzymes: Control by Phosphorylation* 17: 43-111, Academic Press, NY (1986)). Through biochemical studies and gene cloning, four isoforms of the R subunits, RIα, RIβ, RIIα and RIIβ, have been identified (McKnight et al., *Recent Prog. Horm. Res.* 44: 307-335 (1988); and Levy et al., *Molec. Endocrinol.* 2: 1364-1373 (1988)). Three distinct C subunits, Cα, Cβ and Cγ also have been identified (Uhler et al., *PNAS USA* 83: 1300-1304 (1986a); and Uhler

et al., *J. Biol. Chem.* 261: 15360-15363 (1986b); Showers and Maurer, *J. Biol. Chem.* 261: 16288-16291 (1986); and Beebe et al., *Molec. Endocrinol.* 4: 465-475 (1990)); however, preferential co-expression of one of these C subunits with any of the R subunits has not been found (Showers and Maurer (1986), *supra*; Beebe et al. (1990), *supra*). Importantly, the expression of RI/PKA-I and RII/PKA-II has an inverse relationship during ontogenic development and cell differentiation (Lohmann and Walter, In *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* 18: 63-117, Greengard and Robison, eds., Raven Press, NY (1984); and Cho-Chung, *Cancer Res.* 50: 7093-7100 (1990)).

Enhanced expression of the RIα/PKA-I has been shown in human cancer cell lines and in primary tumors, as compared with normal counterparts, in cells after transformation with a chemical or viral carcinogen, the Ki-*ras* oncogene or the transforming growth factor-α, and upon stimulation of cell growth with the granulocyte-macrophage colony-stimulating factor (GM-CSF) or a phorbol ester (Cho-Chung (1990), *supra*; and Miller et al., Eur. J. Cancer 29A(7):989-991 (1993)). Conversely, a decrease in the expression of RIα/PKA-I correlates with growth inhibition induced by site-selective cAMP analogues in a broad spectrum of human cancer cell lines (Cho-Chung et al., *Cancer Inv.* 7: 161-177 (1989)).

The cell surface serves as a key element in many cellular functions, signaling and cell communication, including the cell:cell communication and transduction of signals involved in the regulation of cell growth. This regulation is mediated through receptor molecules and ecto-enzymes which are thought to be modulated either by down regulation, e.g., rapid turnover rate release of proteins from the surface, or by modification of proteins, such as by phosphorylation.

It has now been surprisingly and unexpectedly discovered that an ECPKA exists and that its presence reflects cell transformation resulting from the loss of regulation of cell growth. In view of such a discovery, the present invention seeks to provide a diagnostic and prognostic assay of cancer. Diagnostic and prognostic assays which are convenient, cost-effective, and provide early detection and/or accurate monitoring of cancer are essential to the successful treatment of the disease. However, current diagnostic assays are often burdensome and/or do not provide the

sensitivity and accuracy necessary for early detection of the disease. For example, current methods for diagnosing ovarian cancer involve detecting the presence of protein markers, such as placental alkaline phosphatase and polymorphic epithelial mucin which are associated with ovarian cancer. However, these markers are detected in most women at an advanced stage, when metastatic disease is common and the outcome is almost uniformly fatal since no drug treatment exists for metastasized ovarian cancer. The present inventive diagnostic and prognostic assay seeks to overcome such disadvantages.

It also has been surprisingly and unexpectedly discovered that ECPKA is a measure of hormone-dependence of breast cancer. In view of such a discovery, the present invention seeks to provide a method of determining whether or not breast cancer in a given patient is hormone-dependent or hormone-independent. Current methods of determining hormone-dependent breast cancer involve biopsy and examination of the breast cancer tissue for the presence of estrogen and/or progesterone receptors, which can be detected in the tissue by, for example, an immunohistochemical assay using a monoclonal antibody or by a biochemical assay, such as dextran-coated charcoal. Such methods are disadvantageous because they are inaccurate (as much as 30-40% of results are false positives or false negatives), due to a lack of consensus as to the minimum number of cells required to have an estrogen and/or progesterone receptor for the determination of hormone-dependent cancer, and require biopsy. Accordingly, the present invention seeks to overcome such disadvantages by providing a more accurate assay of the hormone dependency or independency of breast cancer and by not requiring biopsy.

The determination of whether a breast cancer is hormone-dependent or hormone-independent has meaningful implications for the selection of treatment strategy and the prognosis of the disease. For example, if the breast cancer is hormone-dependent, the treatment may include hormone therapy, the prescription of an anti-estrogen drug, or the removal or destruction of ovary function. If the breast cancer is hormone-independent, the treatment will likely include the administration of chemotherapeutic drugs. Furthermore, the absence of estrogen receptors in the primary tumor indicates a higher rate of recurrence and a shorter survival rate.

The present invention additionally provides methods of treating cancer by inhibiting the expression of ECPKA or the type II isozyme of PKA by cancerous cells. These and other objects and advantages, as well as additional inventive features, will be apparent from the detailed description provided herein.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of diagnosing cancer in a patient. The method comprises assaying a sample from the patient for the presence of ECPKA, wherein the presence of an elevated level of ECPKA in the sample compared to the level of ECPKA in a control sample is indicative of cancer in the patient.

The present invention also provides a method of prognosticating cancer in a patient. The method comprises assaying a sample from the patient for the presence of ECPKA, wherein (i) a reduction in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates an improvement in the patient's cancerous condition, (ii) no change in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates no change in the patient's cancerous condition or (iii) an increase in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates a worsening of the patient's cancerous condition.

Also provided by the present invention is a method of determining whether a diagnosed breast cancer is hormone-dependent or hormone-independent. The method comprises assaying a sample from the patient for the presence of ECPKA. An elevated level of ECPKA in the sample as compared to a control sample indicates that the breast cancer is hormone-independent, whereas the presence of a low level of ECPKA in the sample as compared to a control sample indicates that the breast cancer is hormone-dependent.

Additionally, the present invention provides methods for the treatment of cancer. One method comprises reducing the level of ECPKA of cancerous cells by delivering an effective amount of the RIIβ subunit of PKA-II to target cancer cells to down-regulate the expression of ECPKA and inhibit cancer cell growth. Another method comprises inhibiting the expression of the type II isozyme of PKA in cancer

cells by delivering an effective amount of a mutant of the RIα subunit of PKA to target cancer cells to inhibit the expression of the type II isozyme of PKA and induce apoptosis of the cancer cells.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1A is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line.

Fig. 1B is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 1C is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 1D is a bar graph of cell number (x 10^{-6}) vs. cell line.

Fig. 1E is a bar graph of free and total PKA activity (units/mg protein) of T24 bladder carcinoma cell extract vs. time (hours).

Fig. 1F is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of T24 bladder carcinoma cell medium vs. time (hours).

Fig. 1G is a bar graph of LDH activity (mUnits/10^6 cells/ml) of T24 bladder carcinoma cell medium vs. time (hours).

Fig. 1H is a bar graph of cell number (x 10^{-6}) of T24 bladder carcinoma vs. time (hours).

Fig. 2A is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line.

Fig. 2B is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 2C is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 2D is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line.

Fig. 2E is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 2F is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line.

Fig. 3A is a bar graph of free and total PKA activity (units/mg protein) vs. cell line.

Fig. 3B is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. substrate.

Fig. 3C is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. substrate.

Fig. 3D is a line graph of cell number (10^{-5}) vs. time (hours) for a given substrate at a given concentration.

Fig. 3E is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. substrate or inhibitor.

Fig. 3F is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. substrate or inhibitor.

Fig. 3G is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. substrate or inhibitor.

Fig. 4A is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M.

Fig. 4B is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M Cα.

Fig. 4C is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M Cα *mut*.

Fig. 4D is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIα.

Fig. 4E is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIIβ.

Fig. 4F is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M and 8-Cl-cAMP.

Fig. 4G is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIIα.

Fig. 4H is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIIβ-P.

Fig. 4I is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIα-P.

Fig. 5A is a bar graph of PKA activity (mUnits/ml) vs. serum sample from cancer patients.

Fig. 5B is a bar graph of LDH activity (mUnits/ml) vs. serum sample of Fig. 5A.

Fig. 5C is a bar graph of serum PKA (mU/ml) vs. patient treated with the combination of taxotere and GEM231 (RIα antisense) or taxotere alone.

Fig. 6A is a bar graph of growth inhibition (% of control) vs. paclitaxel (nM) for 100 nM RIα antisense, paclitaxel, and the combination of 100 nM RIα antisense and paclitaxel in parental PC3M cells.

Fig. 6B is a bar graph of growth inhibition (% of control) vs. antisense (nM) for 1 nM paclitaxel, RIα antisense, and the combination of 1nM paclitaxel and RIα antisense in parental PC3M cells.

Fig. 6C is a bar graph of growth inhibition (% of control) vs. paclitaxel (nM) for 100 nM RIα antisense, paclitaxel, and the combination of 100 nM RIα antisense and paclitaxel in PC3M RIα-P cells.

Fig. 6D is a bar graph of growth inhibition (% of control) vs. antisense (nM) for 1 nM paclitaxel, RIα antisense, and the combination of 1nM paclitaxel and RIα antisense in PC3M RIα-P cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of diagnosing cancer in a patient. The patient is preferably an animal, more preferably a mammal, most preferably a human.

The diagnostic method comprises assaying a sample from the patient for the presence of ECPKA. An elevated level of PKA in the sample as compared to a control sample is indicative of the presence of cancer.

The method can be used to diagnose any cancer associated with the presence of ECPKA. Whether or not a given type of cancer is associated with the presence of ECPKA can be determined in accordance with the methods set forth herein. Preferably, the cancer is cancer of the breast, ovary, prostate, bladder, colon, pancreas or lung.

While any sample from the patient theoretically can be used in the assay, desirably the sample is a fluid sample. Preferably, the sample is blood, in particular blood serum, or urine. While it is not necessary to assay a control sample every time that a sample from a patient is assayed, it is desirable to do so. The control used can be generated from any type of control sample that allows for a useful comparison with the patient sample. Also, the control can be presented in formats, measurements, or units that are similar to or different from the patient sample as long as a useful comparison can be performed. Preferably, the control sample is in a similar format, measurement and units as the patient sample. For instance, a suitable control is one that is produced from the same biological material using techniques similar to those that are used to generate the patient sample. In this regard, the level of ECPKA in a control sample is from about 0 to about 1.0 mUnits/ml blood serum or urine.

While any method can be used to assay ECPKA as is known in the art, such as the use of [γ-^{32}P] ATP and Kemptide as described in Example 1, desirably ELISA is used. If ELISA is used, an antibody to the catalytic subunit or the regulatory subunit of ECPKA can be used. Irrespective of which method is used, the extent of cell lysis should be assessed. Desirably, cell lysis should be kept to a minimum so that the determination of PKA accurately reflects the amount of ECPKA, not intracellular PKA.

The specific type of cancer detected by the presence of ECPKA can be subsequently or simultaneously determined by methods well-known in the art. Currently, many cancers are associated with well-known genetic or protein markers which are predictive of a particular cancer and which can be detected using well-known assay methods. For example, prostate cancer can be determined by detecting the presence of elevated levels of prostate-specific antigen (PSA) in the blood plasma. Similarly, breast cancer can be determined by detecting elevated levels of such markers as placental isoferritin (p43), carcinoembryonic antigen (CEA), breast cancer associated antigen 15-3 (BRCA 15-3), and laminin. Ovarian cancer may be determined by detecting the presence of M1LP, placental alkaline phosphatase, polymorphic epithelial mucin, and PLAP. Bladder cancer may be determined by detecting the presence of elevated levels of bladder tumor-specific antigen (BTA), basic fibroblast growth factor (bFGF), and cytokeratin-20 (CT-20). Assay methods for determining a specific protein or nucleic acid in a sample are well-known in the art and include such methods as ELISA, Western Blot, Southern Blot, and polymerase chain reaction (PCR) methods, to name a few.

In another embodiment, the present invention provides a method of prognosticating cancer in a patient. The patient is preferably an animal, more preferably a mammal, most preferably a human.

The method comprises assaying a sample from the patient for the presence of ECPKA. A reduction in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates an improvement in the patient's cancerous condition. No change in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates no change in the patient's cancerous condition. An increase in the level of ECPKA in the sample as compared to the level of ECPKA in an earlier sample from the patient indicates a worsening of the patient's cancerous condition. Such a method can be used to assess the resistance of cancer cells to an anti-cancer agent, such as taxol.

While any sample from the patient theoretically can be used in the prognostic assay, desirably the sample is a fluid sample. Preferably, the sample is blood, in particular blood serum, or urine.

While any method can be used to assay ECPKA in the prognostication of cancer as described above with respect to the diagnosis of cancer, desirably ELISA is used. If ELISA is used, an antibody to the catalytic subunit or the regulatory subunit of ECPKA can be used. Cell lysis should be assessed as described above.

In yet another embodiment, the present invention provides a method of determining whether a diagnosed breast cancer is hormone-dependent or hormone-independent. The patient is preferably an animal, more preferably a mammal, most preferably a human.

The method comprises assaying a sample from the patient for the presence of ECPKA. The presence of an elevated level of ECPKA in the sample as compared to a control sample indicates that the breast cancer is hormone-independent. In contrast, the presence of a low level of ECPKA in the sample as compared to the control sample indicates that the breast cancer is hormone-dependent.

While any sample from the breast cancer patient theoretically can be used in the assay, preferably the sample is blood serum.

While any method can be used to assay ECPKA in the determination of the hormone-dependency of breast cancer as described above with respect to the diagnosis of cancer, desirably ELISA is used. If ELISA is used, an antibody to the catalytic subunit or the regulatory subunit of ECPKA can be used. Cell lysis should be assessed as described above.

The present invention additionally provides methods which may be useful for treating cancer by reducing the level of ECPKA of cancerous cells. It has been shown herein that overexpression of the RIIβ subunit of PKA-II leads to the down-regulation of ECPKA and the inhibition of cancer cell growth. Accordingly, one method in which the RIIβ subunit is delivered to target cancer cells may be employed to inhibit the cancer cell growth. Another method in which a mutant of the RIα subunit of PKA, such as a mutant that is mutated at the pseudophosphorylation site of the RIα subunit, such as by introduction of an autophosphorylation site, is delivered to target cancer cells also may be employed to inhibit the expression of both of the wild-type type I and type II isozymes of PKA and induce apoptosis of the cancer cells.

Desirably, the cancer to be treated is cancer of the lung, colon, pancreas, breast, ovary, bladder or prostate.

The general concept of using targeted vectors to deliver a heterologous gene is well-known in the art (Miller et al., FASEB J. 9: 190-199 (1995)). Any suitable vector which is capable of infecting a target cancer cell and expressing the RIIβ subunit or the mutant RIα subunit, as appropriate, in the target cancer cell may be used. Examples of suitable vectors include naked DNA vectors (such as plasmids), viral vectors such as adeno-associated viral vectors (Berns et al., *Annals of the New York Academy of Sciences, 772*, 95-104 (1995)), adenoviral vectors (Bain et al., *Gene Therapy, 1*, S68 (1994)), herpesvirus vectors (Fink et al., *Ann. Rev. Neurosci., 19*, 265-87 (1996)), papilloma virus vectors, picornavirus vectors, polyoma virus vectors, retroviral vectors, SV40 viral vectors, vaccinia virus vectors, and liposomal vectors. Once a given type of vector is selected, its genome must be engineered to incorporate exogenous polynucleotides, including the coding sequence for the RIIβ subunit or the mutant RIα, as appropriate, operably linked to a promoter. Such manipulations are known in the art. Preferably, adeno-associated viral vectors are used.

The vector must target the appropriate cancer cell. Vectors which are modified to target cancer cells by selectively binding to a region on a target cancer-specific, cell-surface molecule are known in the art. For instance, Han et al. (*PNAS USA* 92: 9747-9751 (1995)) discloses the insertion of sequences encoding human heregulin into the envelope of Moloney murine leukemia virus (MoMLV) in order to target the MoMLV virus vector to human breast cancer cells.

Additionally, other vectors can be developed with modifications to bind selectively to cancer-specific, cell-surface molecules by inserting a cancer-specific antibody into the vector which recognizes such molecules. Examples of cancer-specific, cell-surface molecules include, for example, placental alkaline phosphatase (testicular and ovarian cancer), polymorphic epithelial mucin (ovarian cancer), prostate-specific membrane antigen, α-fetoprotein, B-lymphocyte surface antigen (B-cell lymphoma), truncated EGFR (gliomas), gp95/gp97 (melanoma), N-CAM (small cell lung carcinoma), cluster w4, 5A, and 6 (small cell lung carcinoma), CA-125 (lung and ovarian cancers), ESA (carcinoma), CD19, 22 or 37 (B-cell lymphoma), 250 kD

proteoglycan (melanoma), P55 (breast cancer), blood group A antigen in B or O type individual (gastric and colon tumors), PLAP (seminomas, ovarian cancer, and non-small cell lung cancer), and the like. Preferably, the cancer-specific, cell-surface molecules are molecules found in cancerous cells of the breast, the prostate, the ovary or the bladder.

Vectors also can be modified to bind selectively to cancer-specific, cell-surface receptors by inserting a cancer-specific antibody into the vector which recognizes such receptors. Receptors known to be associated with cancer cells include erbB-2 (breast carcinoma), erbB-3, erbB-4, IL-2 (lymphoma and leukemia), IL-4 (lymphoma and leukemia), IL-6 (lymphoma and leukemia), MSH (melanoma), transferrin (gliomas) and tumor vasculature integrins to name a few. Preferably, the cancer-specific, cell-surface receptors are receptors found in breast, prostate, ovarian and bladder cancer cells.

There are a number of antibodies to cancer-specific, cell-surface molecules and receptors that are known. For example, such antibodies include C46 Ab (Amersham) and 85A12 Ab (Unipath) to carcino-embryonic antigen, H17E2 Ab (ICRF) to placental alkaline phosphatase, NR-LU-10 Ab (NeoRx Corp.) to pan carcinoma, RFB4 Ab (Royal Free Hospital) to B-lymphocyte surface antigen, A33 Ab (Genex) to human colon carcinoma, TA-99 Ab (Genex) to human melanoma, antibodies to c-erbB2 (JP 7309780, JP 8176200 and JP 7059588), and the like. Vectors can be developed which specifically target cancer cells, based on such antibodies, using techniques known in the art (see for example, Bind et al., *Science* 242: 423-426 (1988), and Whitlow et al., *Methods* 2(2): 97-105 (1991)).

Alternatively, the vector can be modified to include a ligand for a cancer-specific, cell-surface receptor, or a binding domain for a cancer-specific, cell-surface receptor. Preferably, the vector is modified to include a ligand or binding domain for a cell-surface receptor found on breast, bladder, ovarian or prostate cancer cells. In general, there are a number of databases for ligands, binding domains and cell-surface molecules. See, for example, ftp://kegg.genome.ad.jp, http://broweb.pasteur.fr/docs/versions, http://ampere.doe-mbi.ucla.edu:8801/dat/dip.dat or http://bones.biochem.ualberta.ca/pedro/rt-1.html1.

As mentioned, in one embodiment of the method of treating cancer, the recombinant vector comprises and expresses the coding sequence for the RIIβ subunit within the target cancer cell to produce the subunit in its biologically active form. The coding sequence for the human RIIβ subunit is known (ref. 18 in Levy et al., *Molec. Endocrinol.* 2:1364-1374 (1988)). Expression of the RIIβ subunit in the target cancer cell results in the down-regulation of ECPKA. In the other embodiment of the method of treating cancer, the recombinant vector comprises and expressing a mutant RIα subunit in the target cancer cell. The coding sequence for the human RIα subunit is known. (Sandberg et al., *Biochem. Biophys. Res. Commun.* 149: 939-945 (1987)) Expression of the mutant RIα subunit in the target cancer cell results in inhibition of the expression of both of the wild-type type I and type II isozymes of PKA and induction of apoptosis.

The recombinant vector further comprises a promoter operably linked to the RIIβ coding sequence or the mutant RIα subunit, as appropriate. Additionally, the recombinant vector can include an appropriate enhancer. Any promoter and/or enhancer sequence appropriate for controlling expression of the coding sequences can be used. Such promoter and enhancer elements are well-known in the art. Examples of suitable promoters include prokaryotic promoters and viral promoters (e.g., retroviral ITRs, LTRs, immediate early viral promoters (IEp), such as herpesvirus Iep, cytomegalovirus (CMV) IEp, and other viral promoters, such as Rous Sarcoma Virus (RSV) promoters and Murine Leukemia Virus (MLV) promoters). Other suitable promoters are eukaryotic promoters, such as enhancers (e.g., the rabbit β-globin regulatory elements), constitutively active promoters (e.g., the β-actin promoter, etc.), signal specific promoters including inducible promoters, and tissue- or cell-specific promoters.

If desired, the recombinant vector can be modified such that the transcription of the genome is under the control of a cancer-specific promoter. Preferably, the cancer-specific promoter is one that is only activated in a cell of the cancer that is directly and selectively bound by the recombinant vector. An example of a cancer-specific promoter is CEA. Other promoters can be found on the Internet in the eukaryotic promoter database at http://www.genome.ad.jp/dbget-

bin/www_bFind?epdtable. Alternatively and also preferably, the promoter can be a tissue- or cell-specific promoter, which is active in the tissue from which the cancer is derived. In this regard, preferably the promoter is a tissue-specific promoter which is active in breast cells, ovarian cells, prostate cells or bladder cells.

In view of the above, the present invention provides methods which may be useful for treating cancer in a mammal. "Cancer" according to the invention includes cancers that are characterized by abnormal cellular proliferation and the absence of contact inhibition, which can be evidenced by tumor formation. The term encompasses cancer localized in tumors, as well as cancer not localized in tumors, such as, for instance, cancer that expands from a tumor locally by invasion, or systemically by metastasis. Theoretically, any type of cancer, including lung cancer, kidney cancer, leukemia and the like, can be targeted for treatment according to the invention. Preferably, however, the cancer is breast cancer, ovarian cancer, bladder cancer, or prostate cancer.

The method of treating cancer in a mammal involves the administration to a mammal in need of cancer treatment a cancer treatment effective amount of an above-described recombinant DNA or RNA vector comprising and expressing an effective amount of the RIIβ subunit coding sequence or a mutant RIα subunit coding sequence. Upon entry of the recombinant vector into a cancerous cell, the recombinant vector expresses the RIIβ subunit which inhibits the growth of the cancer or the RIα subunit which leads to induction of apoptosis of the cancer, thereby treating the cancer. Treatment of cancer can be assessed, for example, by monitoring the attenuation of tumor growth and/or tumor regression, wherein "tumor growth" includes an increase in tumor size and/or the number of tumors and "tumor regression" includes a reduction in tumor mass. Desirably, the cancer is a cancer of the breast, ovary, bladder, or prostate.

The present inventive methods of treating cancer in a mammal can be used alone or in combination with radiation, chemotherapy and/or surgery. For example, such combinatorial treatment can be used in the early or late stages of the progression of cancer, including the metastatic stage. For example, the recombinant vector expressing an effective amount of the RIIβ subunit or a mutant RIα subunit in

accordance with the present invention can be introduced into a mastectomy or ovarectomy site, for example, to infect residual tumor cells following surgery. The recombinant vector also can be introduced into the mammary gland by ductal cannulation.

According to the invention, a recombinant vector comprising and expressing the RIIβ subunit coding sequence (or the mutant RIα subunit coding sequence) from which can be transcribed the RIIβ subunit (or the mutant RIα subunit) as described above is administered to a mammal in need thereof. The means of administration of a recombinant vector can be by any suitable means, which, in part, is determined by the type of recombinant vector being administered. For example, a solution comprising a vector may be injected into the tumor mass or by perfusing the blood supply of the tumor. Suitable routes of administration include peritumoral, intratumoral, intravenous, intramuscular, intraperitoneal, subcutaneous, oral, rectal, intraocular, intranasal, and the like. Peritumoral and intratumoral routes of administration, such as by injection, are preferred. Administration by lipofection, direct DNA injection, microprojectile bombardment, liposomes, molecular conjugates and the like, also can be effected. However, the method is not dependent on any particular means of administration and is not to be so construed. Means of administration are well-known to those skilled in the art.

Preferably, the recombinant vector is administered to the mammal in the form of a pharmaceutically acceptable composition. The composition must be such that it does not compromise the ability of the recombinant vector to bind directly and specifically to a cancer-specific, cell-surface molecule or a cancer-specific, cell-surface receptor on the cancer to be treated.

Preferably, the recombinant vector is administered by means of cationic lipids, e.g., liposomes. Such liposomes are commercially available (e.g., Lipofectin®, Lipofectamine™, and the like, supplied by Life Technologies, Gibco BRL, Gaithersburg, MD). Moreover, liposomes having increased transfer capacity and/or reduced toxicity *in vivo* (e.g., as reviewed in PCT patent application no. WO 95/21259) can be employed in the present invention. For liposomal administration, the recommendations identified in WO 93/23569 can be followed. Similarly, other

delivery vehicles include hydrogels and controlled-release polymers. If desired, liposomal formulations and the like can be targeted to cancer cells by causing the liposomes to display an antibody, ligand or binding domain, for example, for a cancer-specific, cell-surface molecule or receptor.

Prior to administration to a mammal, a recombinant vector of the present invention can be formulated into various compositions by combination with appropriate pharmaceutically acceptable carriers or diluents, and can be formulated to be appropriate for either human or veterinary applications.

Thus, a composition for use in the method of the present invention can comprise a recombinant vector preferably in combination with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well-known to those skilled in the art, as are suitable methods of administration. The choice of carrier will be determined, in part, by whether a recombinant alphavirus or a recombinant DNA vector or RNA genome is to be administered, as well as by the particular method used to administer the composition. One skilled in the art will also appreciate that various routes of administering a composition are available, and, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Accordingly, there are a wide variety of suitable formulations of compositions that can be used in the present inventive methods.

A recombinant vector or a composition comprising such vector, alone or in further combination with one or more other active agents, can be made into a formulation suitable for parenteral administration, preferably intraperitoneal administration. Such a formulation can include aqueous and nonaqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and nonaqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use.

Extemporaneously injectable solutions and suspensions can be prepared from sterile powders, granules, and tablets, as described herein.

A formulation suitable for oral administration can consist of liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or fruit juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solid or granules; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers.

Similarly, a formulation suitable for oral administration can include lozenge forms, which can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier; as well as creams, emulsions, gels, and the like containing, in addition to the active ingredient, such carriers as are known in the art.

An aerosol formulation suitable for administration via inhalation also can be made. The aerosol formulation can be placed into a pressurized acceptable propellant, such as dichlorodifluoromethane, propane, nitrogen, and the like.

A formulation suitable for topical application can be in the form of creams, ointments, or lotions.

A formulation for rectal administration can be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate. A formulation suitable for vaginal administration can be presented as a pessary, tampon, cream, gel, paste, foam, or spray formula containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

The dose administered to a mammal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the infected individual over a reasonable time frame. The dose will be determined by the

potency of the particular recombinant vector employed for treatment, the severity of the cancer, as well as the body weight and age of the infected individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the use of the particular recombinant vector employed. It is always desirable, whenever possible, to keep adverse side effects to a minimum.

The dosage can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a vector, alone or in combination with other anticancer agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle. The specifications for the unit dosage forms of the present invention depend on the particular embodiment employed and the effect to be achieved, as well as the pharmacodynamics associated with each compound in the host. The dose administered should be a "cancer treatment effective amount" or an amount necessary to achieve an "effective level" in the individual patient.

Since the "effective level" is used as the preferred endpoint for dosing, the actual dose and schedule can vary, depending on interindividual differences in pharmacokinetics, drug distribution, and metabolism. The "effective level" can be defined, for example, as the blood or tissue level desired in the patient that corresponds to a concentration of one or recombinant vectors according to the invention, which lyses targeted cancerous cells in an assay predictive for clinical anti-cancer activity. The "effective level" for a recombinant vector of the present invention also can vary when the compositions of the present invention are used in combination with other known anti-cancer agents.

One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired "effective level" in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective level" of the compounds of the present invention by a direct (e.g., tumor biopsy or radio-imaging of the tumor) or indirect (e.g., PSA levels in the blood) analysis of appropriate patient samples (e.g., blood and/or tissues).

Further, with respect to determining the effective level in a patient for treatment of cancer, suitable animal models are available and have been widely implemented for evaluating the *in vivo* efficacy against cancer of recombinant DNA protocols (see, e.g., PCR). These models include nude mice and SCID mice. Such models also can be used to evaluate the *in vivo* efficacy of an RNA genome.

Generally, an amount of recombinant sufficient to achieve a tissue concentration of about 10^{-7} M to about 10^{-6} M is preferred. In certain applications, multiple daily doses are preferred. Moreover, the number of doses will vary depending on the means of delivery and the particular recombinant vector administered.

The pharmaceutical composition can contain other pharmaceuticals, in conjunction with a recombinant vector according to the invention, when used to treat cancer therapeutically. In particular, it is contemplated that an anticancer agent be employed, such as, preferably, a recombinant virus, a nucleic acid/liposomal formulation (or other nucleic acid delivery formulation), or another vector system (e.g., retrovirus or adenovirus), either as a viral particle or as a nucleic acid/liposomal formulation. Further representative examples of these additional pharmaceuticals that can be used in addition to those previously described, include chemotherapeutic agents, immunostimulants, antiviral compounds, and other agents and treatment regimes (including those recognized as alternative medicine) that can be employed to treat cancer. Anticancer compounds include, but are not limited to, angiostatin, endostatin, anti-HER-2/neu antibody, and tamoxifen. Immunomodulators and immunostimulants include, but are not limited to, various interleukins, cytokines, antibody preparations, and interferons.

A monoclonal antibody that distinguishes ECPKA from intracellular PKA and ectoPKA can be generated in accordance with methods known in the art. The N-terminal glycine of the Cα subunit of ECPKA may be recognized by a monoclonal antibody. Such a monoclonal antibody would be useful in a kit for carrying out the present inventive methods.

EXAMPLES

The following examples serve to illustrate the present invention and are not intended to limit the scope of the present invention in any way.

Example 1

This example demonstrates the presence of PKA in the conditioned medium of cultured cancer cells.

Cancer cells were maintained in appropriate growth medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM minimum essential medium (MEM) non-essential amino acids, pH 7.4, and antibiotic-antimycotic in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. For PKA enzyme assays, cells were seeded at a density of 2-7x10^5 cells per 60 mm plate. When the cells were about 50-60% confluent, the culture medium was removed and fresh medium (2 ml) was added. After 24 hours of incubation, the conditioned medium was collected and cells were harvested for PKA assays.

After harvesting by scraping and centrifugation, cell pellets were washed in NaCl/Pi buffer (0.0017 M KH_2PO_4, 0.005 M Na_2HPO_4, 0.15 M NaCl, pH 7.4). The final cell pellets were suspended in 500 µl buffer 10 (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1% Nonidet P-40 (NP40), 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.4 mg/ml aprotinin and 0.5 mg/ml soybean trypsin inhibitor filtered through a 0.45-µm pored membrane), passed through a 20-gauge needle five times using a 1-ml syringe, allowed to sit at 4 °C for 15 min and then centrifuged for 5 min in an Eppendorf microfuge at 4 °C. The supernatant was used as cell extract. Protein concentration (usually 1-5 mg/ml) was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

The enzyme activity of PKA was measured by a previously described method (Rohlff et al., J. Biol. Chem. 266(8): 5774-5782 (1993)). For the measurement of conditioned medium PKA activity, the assays were carried out using 200 µl of medium for 20 min at 37 °C. The reaction mixture (total volume, 250 µl) contained 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 5 µM Kemptide (a

serine-containing peptide that carries the specific recognition and phosphorylation sites for PKA; Leu-Arg-Arg-Ala-Ser-Leu-Gly [SEQ ID NO: 1], GIBCO-BRL, Gaithesburg, MD)), 1.2 μM [γ-^{32}P] (25 Ci/mmol, ICN, Costa Mesa, CA) with or without 5 μM cAMP (so as to measure the free C subunit activity and the total kinase activity) and 5 μM PKI (Walsh-Krebs inhibitor, an inhibitory protein that is specific for PKA). For cell extract PKA measurement, the assays (total volume, 50 μl) were carried out for 5 min at 37 °C in the reaction mixture (see above) containing 10 μg protein. After incubation, the reaction mixtures were spotted onto phosphocellulose disks (GIBCO-BRL) and were washed three times in 0.5% phosphoric acid. Filters were air-dried and then counted by liquid scintillation counter (Beckman, Fullerton, CA). One unit of enzyme is defined as the amount of enzyme that will transfer 1.0 pmol of phosphate from [γ-^{32}P]ATP to Kemptide per min at standard assay conditions. In parallel with the ECPKA assay of the conditioned medium, the intracellular PKA of the cell extracts prepared from the cells of the same culture from which the conditioned medium was obtained was also measured.

Lactate dehydrogenase activity was measured by the use of a commercial kit (Sigma Chemical Co., St. Louis, MO). Briefly, 25 μl of conditioned medium were added to 250 μl of pyruvate-NADH-containing reaction mixture. After a 30-min incubation at 37 °C, 250 μl of 2,4-dinitrophenylhydrazine (dissolved in 1 N HCl, 20 mg/dL) were added to the reaction mixture and the reactions were allowed to stand at room temperature for 20 min. Sodium hydroxide (2.5 ml of 0.4 N solution) was then added to each reaction mixture and mixed thoroughly by the use of vortex. The absorbance of reaction mixtures was measured at 464 nm.

As shown in **Fig. 1B**, which is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line, varying degrees of ECPKA activity were detected in the conditioned medium from cancer cell lines of various cell types including lung (A549 (American Type Culture Collection (ATCC), Rockville MD)), bladder (J82 (ATCC), T24 (ATCC), UMUC3 (ATCC)), colon (HCT-15 (National Cancer Institute (NCI) Frederick Cancer Research Facility, Frederick, MD)), CoLo205 (NCI), LS-174T (John W. Grainer, NCI, Bethesda, MD), and kidney (293 (Kenneth H. Cowan, NCI, Bethesda, Md)), 293T (Kenneth H. Cowan, NCI, Bethesda,

MD)) carcinoma cells. The PKA activity detected in the conditioned medium was not activated by exogenously added cAMP (**Fig. 1B**). Thus, the ECPKA activity represents the free C subunit activity. This is in sharp contrast with the intracellular PKA activity in the cell extracts. As shown in **Fig. 1A**, which is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line, there was almost no extracellular free C activity, and only in the presence of exogenous cAMP was intracellular PKA detected, indicating that the intracellular PKA was exclusively present in an inactive holoenzyme form. Importantly, the pattern of ECPKA activity detected in conditioned medium of different cell lines did not correlate with the intracellular PKA activity, the conditioned medium-LDH activity (**Fig. 1C**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line) or the cell number in the culture dish (**Fig. 1D**, which is a bar graph of cell number (x 10^{-6}) vs. cell line).

Example 2

This example describes the temporal course of ECPKA accumulation in the conditioned medium of bladder carcinoma cells.

The temporal accumulation of ECPKA in the conditioned medium of bladder carcinoma cells was assayed in accordance with the methods set forth in Example 1. As shown in **Fig. 1F**, which is a graph of free PKA activity (mUnits/10^9 cells/ml) of T24 bladder carcinoma cell medium vs. time (hours), the ECPKA increased in the conditioned medium in a time-dependent manner. The kinase activity showed a peak activity at 12 h of culture and plateaued thereafter up to 24 h. At 48 h, the PKA activity further increased showing a biphasic curve of activity. This pattern of the time-dependent increase of the ECPKA was similar to that of intracellular PKA (**Fig. 1E**, which is a bar graph of free and total PKA activity (units/mg protein) of T24 bladder carcinoma cell extract vs. time (hours)) and cell number increase (**Fig. 1H**, which is a bar graph of cell number (x 10^{-6}) of T24 bladder carcinoma vs. time (hours)). Thus, accumulation of ECPKA was a function of cell growth and intracellular PKA. Because the LDH activity sharply increased at 48 h of culture (**Fig. 1G**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of T24 bladder

carcinoma cell medium vs. time (hours)), ECPKA activity was measured at 24 h of cell culture to avoid any non-specific cell damage-related excretion of PKA.

Example 3

This example demonstrates that ECPKA expression is inversely related to hormone dependency in breast cancer cells.

The conditioned media from the 24 h culture of hormone-dependent (MCF-7 (ATCC), T-47D (David Salomon, NCI, Bethesda, MD)), and hormone-independent (SK-BR-3 (David Salomon, NCI, Bethesda, MD), MDA-MB-231 (ATCC)), and hormone-dependent/multi-drug resistant (MCF-7^{TH} (Susan Bates, NCI, Bethesda, MD)) breast cancer cells were assayed for ECPKA activity. The ECPKA of these breast cancer cells was present in active, free C subunit form, as shown in **Fig. 2B**, which is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line, whereas the intracellular PKA was present in inactive holoenzyme form in these breast cancer cells, as shown in **Fig. 2A**, which is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line, and in other cancer cell lines, as shown in **Fig. 1A**. The hormone-independent breast cancer cells had higher levels of ECPKA than the hormone-dependent breast cancer cells (**Fig. 2B**). This pattern of ECPKA expression paralleled intracellular PKA expression in these cells with the exception of MCF-7TH cells, which showed an inverse relation between intracellular and ECPKA (**Fig. 2A** and **Fig. 2B**). There was no correlation between LDH activity in the conditioned medium of these cells and the ECPKA in these cells as shown in **Fig. 2C**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line. These results support an inverse relationship between ECPKA expression and hormone-dependency in breast cancer cells.

Example 4

This example demonstrates that ECPKA expression is independent of prostate-specific antigen (PSA) expression.

Given that prostate-specific antigen (PSA) determination has been used for diagnosis of prostate cancer, ECPKA expression was examined in prostate cancer

cells that express low and high levels of PSA. As shown in **Fig. 2E**, which is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. cell line, the ECPKA levels measured in the conditioned medium of four different prostate cancer cell lines were 100-180-fold greater than that of the immortalized prostate epithelial cell line PrEC5500 (Clonetics, San Diego, CA). Thus, these prostate cancer cells exhibited a high level of ECPKA regardless of their PSA levels. Importantly, the immortalized PrEC5500 cells contained intracellular PKA at a level comparable to that in prostate cancer cells, as shown in **Fig. 2D**, which is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. cell line, but exhibited very low levels (0.2 mUnits/10^6 cells/ml) of ECPKA as shown in **Fig. 2E**. The patterns of ECPKA expression of these prostate cancer cell lines were distinctive from their intracellular PKA expression patterns, and were unrelated to LDH expression (**Fig. 2F**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. cell line).

Example 5

This example demonstrates that expression of ECPKA is regulated by intracellular PKA.

Given that protein kinase A isozyme type I, as opposed to type II, has been implicated in cell transformation (Cho-Chung, *Cancer Res.* 50: 7093-7100 (1990)), whether or not PKA isozyme distribution in the cell contributes to ECPKA was examined using site-selective cAMP analogs, which can differentially regulate PKA isozymes (Doskeland, *Biochem. Biophys. Res. Commun.* 83: 543-549 (1978); and Rannels and Corbin, *J. Biol. Chem.* 255: 7085-7088 (1980)). One such analog, 8-Cl-cAMP, which possesses a higher affinity for RI of both site A and site B than parental cAMP, can efficiently dissociate PKA-I holoenzyme into RI and C subunits leading to down regulation of PKA-I without affecting PKA-II (Cho-Chung, 1990).

PC3M cells (NCI Federick Cancer Research Facility, Federick, MD) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM MEM nonessential amino acids, pH 7.4, and antibiotic-

antimycotic in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Cells were seeded at a density of 1 x 10^5 cells/60 mm dish, the cAMP analogue 8-Cl-cAMP (5 μM, 3 days) or 8-Cl-adenosine (2 μM, 3 days) was added and then the intracellular (in cell extracts) and extracellular (in conditioned medium) PKA activities were measured in accordance with the methods set forth in Example 1. As shown in **Fig. 3A**, which is a bar graph of free and total PKA activity (units/mg protein) vs. cell line, and **Fig. 3B**, which is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. substrate, 8-Cl-cAMP downregulated both intracellular PKA and ECPKA. As shown in **Fig. 3D**, which is a line graph of cell number (10^{-5}) vs. time (hours) for a given substrate at a given concentration, 8-Cl-cAMP also induced growth inhibition. The cytotoxic metabolite, 8-Cl-adenosine, which brought about a marked inhibition of cell growth (**Fig. 3D**) and increased LDH activity in the conditioned medium as shown in **Fig. 3C**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium vs. substrate, had a minimal effect on the intracellular PKA or ECPKA levels (**Figs. 3A** and **3B**). DEAE-column chromatography showed that these effects of 8-Cl-cAMP on PKA inhibition clearly result in the selective downregulation of PKA-I isozyme, as shown in **Fig. 4F**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M and 8-Cl-cAMP.

Given that alterations in PKA isozyme distribution in cancer cells can be brought about by overexpression of the regulatory and catalytic subunit genes of PKA (Tortora and Cho-Chung, *J. Biol. Chem.* 265: 18067-18070 (1990); and Nesterova et al., *Eur. J. Biochem.* 235: 486-494 (1996)), PC3M cells were transfected with Cα, Cα mutant, RIα, and RIIβ genes in the metal ion-inducible (MT-expression) vector OT1521/OT1529 (McGeady et al., *Oncogene* 4: 1375-1382 (1989)). The Cα mutant gene was generated by subcloning a *Bam* HI/*Sal* I fragment containing the complete open frame of human Cα cDNA (Steven K. Hanks, The Salk Institute, San Diego, CA) (Maldonado and Hanks, *Nucleic Acids Res.* 16: 8189-8190 (1998)) into the vector pGEX-4T-1 (Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ) and introducing two mutations into the gene (Kamps et al., *Cell* 46: 105-112 (1988)), thereby altering the NH_2 terminal Gly (GGC) to an Ala (GCA), by using the site-

directed mutagenesis system (Stratagene, La Jolla, CA; Catalog No. 200518). The following primers were used (mutation underlined):

5'-ccg-cgt-gga-tcc-**atg-gca-aac-gcc-gcc-gcc**-3' [SEQ ID NO: 2] and

5'-**ggc-ggc-ggc-ggc-gtt-tgc-cat**-gga-tcc-acg-cgg-3' [SEQ ID NO: 3]. DNA sequencing analysis verified that no additional mutations were introduced. The *Bam* HI/*Not* I of pGEX-Cα (wild-type or mutant) vector fragment was inserted into the vector pcDNA 3.1 (Invitrogen, Carlsbad, CA) and then the *Hin* dIII/*Xba* I fragment was inserted into the vector pGEM-11zf(+) (Promega, Madison, WI). Finally, the pGEM-11zf(+) Cα vector was cut with *Bam* HI, and the fragment was cloned into the *Bam* HI site of the vector OT1529 (McGeady et al. (1989), *supra*) to produce the retroviral vector MT-1 (Tortora and Cho-Chung (1991), *supra*).

PC3M cells (10^6 cells/100 mm plate) were transfected with 7.5 μg of the MT-expression vector plasmid containing Cα, C*αmut* , RIα or RIIβ subunits of PKA by the lipofectin method (GIBCO-BRL). Forty eight hours after transfection, the neomycin analog G418 (400 μg/ml) was added to the medium, and resistant colonies were isolated 2-3 weeks after selection. Colonies were grown in the presence of 60 μM $ZnSO_4$ for 6 days and were examined for their expression. Clones that overexpressed the gene were pooled and used for the experiments.

Clones were selected for overexpression of each transfected gene and examined for intracellular and ECPKA levels

Cells overexpressing Cα exhibited a 3.5-fold increase in intracellular PKA (cell extract) as shown in **Fig. 3E**, which is a bar graph of free and total PKA activity (units/mg protein) of cell extract vs. substrate or inhibitor, and a 6-fold increase in extracellular (conditioned medium) PKA as shown in **Fig. 3F**, which is a bar graph of free PKA activity (mUnits/10^6 cells/ml) of cell medium vs. substrate or inhibitor. RIα overexpression led to a 3-fold increase in the intracellular PKA as shown in **Fig. 3E** and a 5-fold increase in the ECPKA as shown in **Fig. 3F**. This increase in ECPKA was not due to cell damage because the level of LDH in the conditioned medium remained the same as that in the untransfected parental cells (as shown in **Fig. 3G**, which is a bar graph of LDH activity (mUnits/10^6 cells/ml) of cell medium. DEAE-column chromatography analysis showed that both Cα and RIα overexpression led to

a marked increase in the level of type I PKA holoenzyme without affecting the level of type II PKA as shown in **Fig. 4B,** which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M Cα (Fig. 4A is corresponding line graph for the control, nontransfected parental cells PC3M), and **Fig. 4D**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIα, respectively. Overexpression of RIα-P (an RIα mutant at the pseudophosphorylation site by introduction of an autophosphorylation site by a point mutation of G to T in the first nucleotide of the codon encoding amino acid 99, thereby converting alanine to serine) did not increase much ECPKA over that of parental cells, even though the cells were capable of upregulating intracellular PKA-I (see **Figs. 3E** and **3F** and **Fig. 4I**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIα-P). RIIα overexpression, which slightly increased PKA type II (see **Fig. 4G**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIIα), did not alter ECPKA levels (see **Figs. 3E** and **3F**). Overexpression of RIIβ-P (an RIIβ mutant at the autophosphorylation site by introduction of a point mutation of T to G in the first nucleotide of the codon encoding amino acid 114, thereby converting serine to alanine), which did increase PKA type II (see **Fig. 4H**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M RIIβ-P), also did not increase ECPKA over that of parental cells (see **Figs. 3E** and **3F**).

In contrast, RIIβ overexpression led to no change in intracellular PKA level and, markedly, a reduction in the ECPKA expression as shown in **Fig. 3E** and **Fig. 3F**, respectively. DEAE-column chromatography showed that RIIβ overexpression markedly down-regulated PKA-I, along with upregulation of PKA-II, as shown in **Fig. 4E**, which is a line graph of PKA activity (units/50 μl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP

and PKI for PC3M RIIβ. Importantly, overexpression of RIIβ brought about changes in cell morphology (as determined by washing cells in PBS, fixing cells with 70% methanol for 5 min, staining with Giemsa (Bio-Rad, Sigma Chemical Co.) for 15 min and visualizing under an inverted microscope) to that of typical flat phenotype and retarded cell growth, whereas Cα- or RIα-overexpressing cells exhibited no change in cell morphology or cell growth.

The following table, Table I, summarizes the above results.

Table I

PC3M

Cell Line	Cell Extract				Conditioned Medium	
	Total PKA Activity (U/mg protein)	Free PKA Activity (U/mg protein)	PKA Activity Ratio -cAMP / +cAMP	%	Free PKA Activity ($mU/10^6$ cells/ml)	LDH Activity ($mU/10^6$ cells/ml)
Parent	0.84 ± 0.099	0.009 ± 0.00018	0.011	100	29.8 ± 6	36.2 ± 2.6
Cα	3.09 ± 0.562	0.033 ± 0.0054	0.011	100	157.2 ± 28	42.4 ± 1.5
Cαmut	3.15 ± 0.542	0.035 ± 0.0065	0.011	100	43.6 ± 22	39.2 ± 9
RIα	2.84 ± 0.015	0.015 ± 0.0016	0.005	48	122.6 ± 30	44.5 ± 3.7
RIα-p	1.92 ± 0.033	0.033 ± 0.0018	0.017	155	65.0 ± 8	48.0 ± 5.7
RIIα	1.40 ± 0.266	0.006 ± 0.0017	0.004	39	32.6 ± 12	47.7 ± 5.1
RIIβ	0.94 ± 0.112	0.005 ± 0.0015	0.005	48	13.6 ± 4	54.5 ± 3.7
RIIβ-p	1.83 ± 0.465	0.007 ± 0.002	0.004	35	55.0 ± 8	45.1 ± 4.1

The following table, Table II, summarizes what upregulates and downregulates ECPKA.

Table II

ECPKA

Upregulation	Downregulation
Increase in type I PKA	Increase in type II PKA
RIα overexpression	RIIβ overexpression
Cα overexpression	Cα-*mut* overpression
Promotion of cell proliferation (Growth factors, Oncogenes)	RIα-*pmut* overexpression
Promotion of cell survival (Bcl-2 upregulation)	RIα antisense ODN or RIα antisense gene overexpression
Multidrug Resistance	Programmed cell death
Transformed phenotype	Reverted phenotype

Example 6

This example demonstrates that prevention of myristylation of the catalytic subunit of PKA blocks ECPKA expression.

The catalytic (C) subunit of PKA is acylated at its amino terminus with myristic acid (Carr et al., *PNAS USA* 79: 6128-6131 (1982)). In the C subunit of sperm (Cs), the amino-terminal myristate and the first 14 amino acids of Cα are replaced by an amino-terminal acetate and six different amino acids (San Agustin et al., *J. Biol. Chem.* 38: 24874-24888 (1998)). It has been suggested that this different amino terminus of Cα may be related to a unique requirement for localization of the "free" C subunit within the sperm flagellum (San Agustin et al. (1998), *supra*). In view of this, the possible role of C subunit myristylation with respect to ECPKA was examined using the cDNA expression vector OT1529 (McGeady et al. (1989), *supra*), in which the acylated amino-terminal Gly was mutagenized to Ala.

As shown in **Fig. 3E**, the mutant Cα, Cα-ala-overexpressing cells markedly increased the intracellular PKA levels to the same extent as the wild-type Cα-overexpressing cells. However, unlike the wild-type Cα-overexpressing cells, which markedly increased the ECPKA level, the mutant Cα-ala-overexpressing cells were unable to increase the ECPKA level above that of non-transfected parental cells (**Fig. 3F**).

In preparation for DEAE-column chromatography, cell pellets (4 x 10^7 cells) were washed two times with ice-cold NaCl/Pi buffer, were suspended in 15 ml of 10 mM Tris/HCl, pH 7.1, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1mM benzamidine, 30 μg/ml leupeptin, 5.0 μg/ml aprotinin, and 5.0 μg/ml pepstatin and were kept on ice for 30 min. The cells were homogenized (70 strokes) with a Dounce homogenizer, were centrifuged at 10,000 g for 20 min and were filtered through a 0.45-μm pored syringe filter. The supernatants were collected and assayed for protein concentration using Bradford assay (Bio-Rad), and were used as the cell extracts for chromatography. The DEAE column (0.9 x 5.0 cm) was equilibrated with Buffer A (10 mM Tris/HCl, pH 7.1, containing 1 mM EDTA and 1 mM PMSF). Cell extracts (10 mg protein) were loaded onto the column, which was washed with 30 ml of Buffer A and was eluted with a 0 to 0.4 M NaCl gradient in Buffer A with a 1.4 ml fraction volume. PKA assay (total volume 100 μl) was carried out as described in Example 1 using 50 μl of column fractions.

DEAE-column chromatography analysis showed that the mutant Cα-ala cells were capable of inducing PKA-I holoenzyme level to the same extent as the wild- type Cα cells, as shown in **Fig. 4B** and **Fig. 4C**, which is a line graph of PKA activity (units/50 µl) vs. fraction number vs. NaCl concentration (M) in the presence of cAMP alone or in the presence of cAMP and PKI for PC3M Cα *mut*. These results indicate that N-terminal myristylation is an essential requirement for C subunit excretion to the extracellular space.

Example 7

This example demonstrates that ECPKA is immunologically related to intracellular PKA.

The parental and transfected PC3M cells were grown in the absence or presence of 60 µM $ZnSO_4$. Cell extracts were prepared as described in Example 1. For detection of PKA subunits in conditioned medium, 10 ml culture medium of PC3M cells was concentrated 150 times with microcon (Millipore, Bedford, MA). Ten µg protein from cell extracts or 20 µl of concentrated medium were subjected to SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk and 1% BSA for 1 hour at 4°C and were probed with monoclonal antibodies to Cα, RIα or RIIβ (Pharmingen/Transduction Laboratories, San Diego, CA) for 4 hours at 4°C. Blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the Amersham ECL™ system (Amersham, Pharmacia Biotechnology, Inc.).

Probing with anti-human Cα antibody identified the presence of Cα protein in cell extract and conditioned medium. The Cα protein from cell extract and conditioned medium co-migrated the same distance in SDS-PAGE exhibiting a single protein band of 40 kDa. When probed with anti-human RIα antibody, a single protein band of 48 kDa was detected in cell extract and conditioned medium. RIIα and RIIβ were detected only in the cell extract but not in the conditioned medium. These results indicate that the ECPKA is a type I PKA.

Example 8

This example demonstrates the presence of ECPKA in the serum of cancer patients.

Serum samples were obtained from cancer patients with a variety of cancers, including renal, colon, rectum and skin carcinomas and melanomas and were assayed for

LDH activity (using 10 µl of six-fold diluted serum) and PKA activity (using 10 µl serum) as described in Example 1. **Fig. 5A**, which is a bar graph of PKA activity (mUnits/ml) vs. serum sample, shows the data from normal patients (n=49), patients without cancer (n=16), and patients suffering from breast (n=5), colon (n=40), lung (n=6), melanoma (n=131), ovarian (n=7), pancreas (n=6), rectal (n=9), renal cell (n=78), other carcinomas (n=68) and total carcinomas (n=348). The ECPKA activity was significantly elevated in the serum samples of cancer patients as compared to that in normal serum samples. The mean ±S.D. value of PKA activity (mU/ml) in the sera of cancer patients was 76.7 ± 30.1 (range: 25.1 - 311.2; n=348), while in normal persons (control) and patients with no cancer the PKA means ± S.D. were 11.0 ± 5.7 (range: 1.9 - 47.6; n=91) and 6.6 ± 2.5 (range: 4.0 - 10.1; n=16), respectively as shown in Table III.

Table III

#	Designation	n	Mean	SD	Lowest	Highest
1	Normal Control	91	11.0	5.7	1.9	47.6
2	Patient w/o cancers	16	6.6	2.5	4.0	10.1
3	***Total***	***348***	***76.7***	***30.7***	***25.1***	***311.2***
4	Breast	5	185.5	180.0	57.8	311.2
5	Colon	40	110.0	33.4	26.3	146.9
6	Melanoma	91	121.2	44.0	27.9	167.7
7	Ovary	7	105.5	14.4	46.6	110.7
8	Rectum	9	85.6	21.4	26.8	106.1
9	Renal Cell	68	114.7	58.4	25.1	164.3
10	Lung	6	109.9	32.4	34.1	121.0
11	Pancreas	6	200.9	129.4	31.0	303.1
12	Others	116	106.5	39.4	28.1	123.1

Further, the ECPKA detected in the human sera was not stimulated with cAMP, but was inhibited by the PKA inhibitor, PKI. This indicates that the ECPKA in the human sera was present in the active, "free" C subunit form.

The LDH levels of all samples were within comparable values of 148 – 158 mU/ml (normal range: 55-170 mUnits/ml) as shown in **Fig. 5B**, which is a bar graph of LDH activity (mUnits/ml) vs. serum sample, indicating no significant cell degradation in these serum samples.

The ECPKA levels were downregulated in the serum of cancer patients after treatment with taxotere or the combination of taxotere and GEM 231 (RIα antisense) as shown in **Fig. 5C**, which is a bar graph of serum PKA (mU/ml) vs. patient treated with the combination of taxotere and GEM231 (RIα antisense) or taxotere alone.

Example 9

This example demonstrates the effect of RIα antisense and paclitaxel on ECPKA of PC3M cells and PC3M RIα-P mutant cells.

PC3M cells and PC3M RIα-P mutant cells were exposed to paclitaxel, RIα antisense, or the combination of paclitaxel and RIα antisense. The results are shown in **Figs. 6A-6D**. **Fig. 6A** is a bar graph of growth inhibition (% of control) vs. paclitaxel (nM) for 100 nM RIα antisense, paclitaxel, and the combination of 100 nM RIα antisense and paclitaxel in parental PC3M cells, whereas **Fig. 6B** is a bar graph of growth inhibition (% of control) vs. antisense (nM) for 1 nM paclitaxel, RIα antisense, and the combination of 1nM paclitaxel and RIα antisense in parental PC3M cells, **Fig. 6C** is a bar graph of growth inhibition (% of control) vs. paclitaxel (nM) for 100 nM RIα antisense, paclitaxel, and the combination of 100 nM RIα antisense and paclitaxel in PC3M RIα-P cells, and **Fig. 6D** is a bar graph of growth inhibition (% of control) vs. antisense (nM) for 1 nM paclitaxel, RIα antisense, and the combination of 1nM paclitaxel and RIα antisense in PC3M RIα-P cells. The results are summarized in Table IV.

Table IV

Cell Line	Cell Extract				Conditioned Medium	
	Total PKA Activity (U/mg protein)	Free PKA Activity (U/mg protein)	PKA Activity Ratio -CamP / +cAMP	%	Free PKA Activity ($mU/10^6$ cells/ml)	LDH Activity ($mU/10^6$ cells/ml)
Parent PC3M	0.84	0.009	0.011	100	29.8	36.2
+ Antisense	0.71	0.010	0.014	127	22.2	45.3
+ Paclitaxel	0.75	0.011	0.015	136	23.0	41.1
+Antisense/ paclitaxel	0.68	0.012	0.018	164	20.1	42.1
PC3M $_{RIa-p}$	1.92	0.033	0.017	155	65.0	48.0
+ Antisense	1.32	0.030	0.023	209	45.0	49.3
+ Paclitaxel	1.72	0.034	0.020	181	58.8	48.1
+Antisense/ paclitaxel	1.29	0.030	0.023	209	42.5	50.1

Thus, in view of the above, these results show that both of an antisense targeted against PKA RIα subunit and a microtuble-damaging drug, paclitaxel, down-regulated ECPKA expression in PC3M prostate cancer cells (Table III). These results explain the clinical data of taxotere alone or taxotere plus Gem231 (RIα AS) treatment that brought about marked down-regulation of ECPKA levels in the serum of cancer patients (Fig. 5C). The down- regulation of ECPKA by RIα-AS and/or paclitaxel treatment in PC3M cells was inversely related to the PKA activity ratio, which measures the activated free catalytic (C) subunit of PKA, of intracellular PKA (Table III). Thus, the ECPKA present in active, free C subunit form is not merely a reflection of PKA activation, namely an increase in the free C subunit, in the cell. Cells overexpressing the mutant RIα-P, which contain no type II PKA, mimicking lymphoma cells, expressed greater amount of ECPKA and were rather resistant to paclitaxel treatment compared to parental PC3M cells (Table III). Thus, ECPKA measurement can be used to determine taxol resistance of cancer cells.

The documents cited herein are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred assays may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of diagnosing cancer in a patient which method comprises assaying a sample from said patient for the presence of extracellular cAMP-dependent protein kinase (ECPKA), wherein the presence of an elevated level of ECPKA in said sample compared to the level of ECPKA in a control sample is indicative of cancer in said patient.

2. The method of claim 1, wherein said sample is blood serum.

3. The method of claim 2, wherein the level of ECPKA in said control sample is from about 0 to about 1.0 mUnits/ml blood serum.

4. The method of claim 2, wherein said cancer is breast cancer, prostate cancer, ovarian cancer, colon cancer, pancreatic cancer, lung cancer, or bladder cancer.

5. The method of claim 1, wherein said sample is urine.

6. The method of claim 4, wherein the level of ECPKA in said control sample is from about 0 to about 1.0 units/ml urine.

7. The method of claim 1, wherein said assaying involves the use of ELISA.

8. The method of claim 7, wherein said ELISA involves the use of an antibody to the catalytic subunit of ECPKA.

9. The method of claim 7, wherein said ELISA involves the use of an antibody to the regulatory subunit of ECPKA.

10. A method of determining the hormone dependency of breast cancer in a patient, which method comprises assaying a sample from said patient for the presence of ECPKA, wherein the presence of an elevated level of ECPKA in said sample compared to the level of ECPKA in a control sample is indicative of hormone-independent breast cancer in said

patient and wherein the presence of a low level of ECPKA in said sample compared to the level of ECPKA in a control sample is indicative of hormone-dependent breast cancer in said patient.

11. A method of prognosticating cancer in a patient, which method comprises assaying a sample from said patient for the presence of ECPKA, wherein (i) a reduction in the level of ECPKA in said sample as compared to the level of ECPKA in an earlier sample from said patient indicates an improvement in the patient's cancerous condition, (ii) no change in the level of ECPKA in said sample as compared to the level of ECPKA in an earlier sample from said patient indicates no change in the patient's cancerous condition or (iii) an increase in the level of ECPKA in said sample as compared to the level of ECPKA in an earlier sample from said patient indicates a worsening of the patient's cancerous condition.

12. The method of claim 11, wherein said sample is blood serum.

13. The method of claim 12, wherein said cancer is breast cancer, prostate cancer, ovarian cancer, colon cancer, pancreatic cancer, lung cancer, or bladder cancer.

14. The method of claim 11, wherein said sample is urine.

15. The method of claim 11, wherein said assaying involves the use of ELISA.

16. The method of claim 15, wherein said ELISA involves the use of an antibody to the catalytic subunit of ECPKA.

17. The method of claim 15, wherein said ELISA involves the use of an antibody to the regulatory subunit of ECPKA.

18. A method of treating cancer in a patient by inhibiting the expression of ECPKA, which method comprises administering to said patient a recombinant vector that is targeted to

cancer cells and expresses an effective amount of the RIIβ subunit of PKA in said cancer cells, whereupon the expression of ECPKA is inhibited.

19. A method of treating cancer in a patient by inhibiting the expression of the wild-type type I and type II isozymes of PKA, which method comprises administering to said patient a recombinant vector that is targeted to cancer cells and expresses an effective amount of a mutant of the RIα subunit of PKA in said cancer cells, whereupon the expression of both of the wild-type type I and type II isozymes of PKA in said cancer cells are inhibited and apoptosis of the cancer cells is induced.

20. The method of claim 19, wherein said mutant is characterized by the introduction of an autophosphorylation site into the pseudophosphorylation site of said RIα subunit of PKA.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/79281 A1

(51) International Patent Classification[7]: G01N 33/574, 33/573, A61K 48/00

(21) International Application Number: PCT/US00/16628

(22) International Filing Date: 16 June 2000 (16.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/140,288 18 June 1999 (18.06.1999) US

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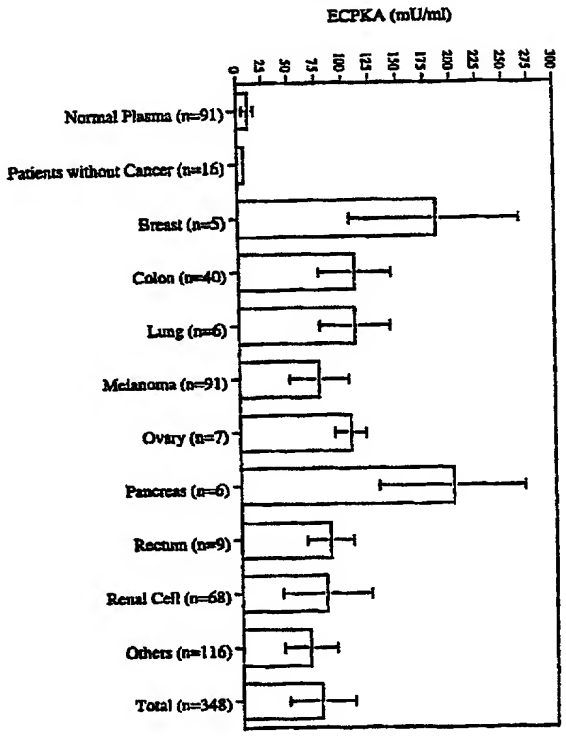
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

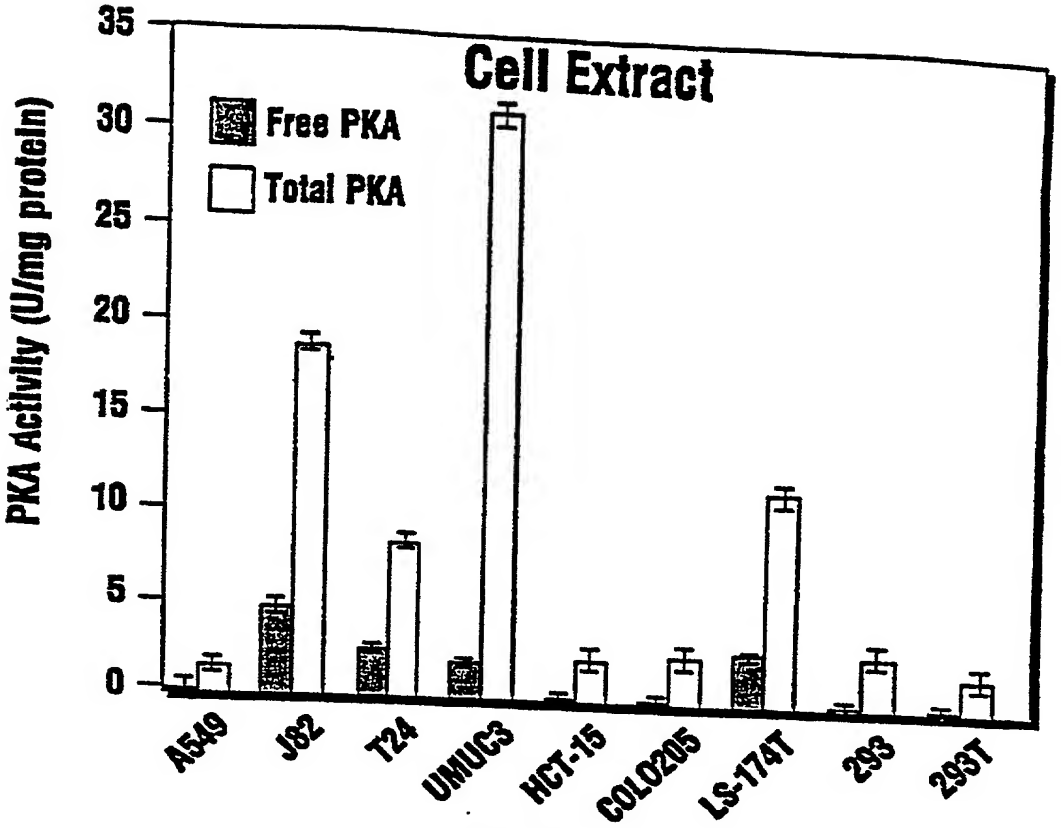
[Continued on next page]

(54) Title: EXTRACELLULAR CAMP-DEPENDENT PROTEIN KINASE IN DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER



(57) Abstract: The present invention provides methods for diagnosing and prognosticating cancer in a patient as well as a method of determining whether or not a diagnosed breast cancer is hormone-dependent or hormone-independent. The methods comprise assaying a sample from a patient for ECPKA (ECPKA). Also provided by the present invention are a method that is potentially useful in treating cancer in a patient by reducing the level of ECPKA by down-regulation, such as by delivering the RIIβ subunit of PKA-II to target cancer cells, and a method that is potentially useful in treating cancer in a patient by inhibiting the expression of both of the wild-type type I and type II isozymes of PKA, such as by delivering a mutant of the RIα subunit of PKA to target cancer cells.

WO 00/79281 A1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Free PKA
Total PKA
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30
25
20
15
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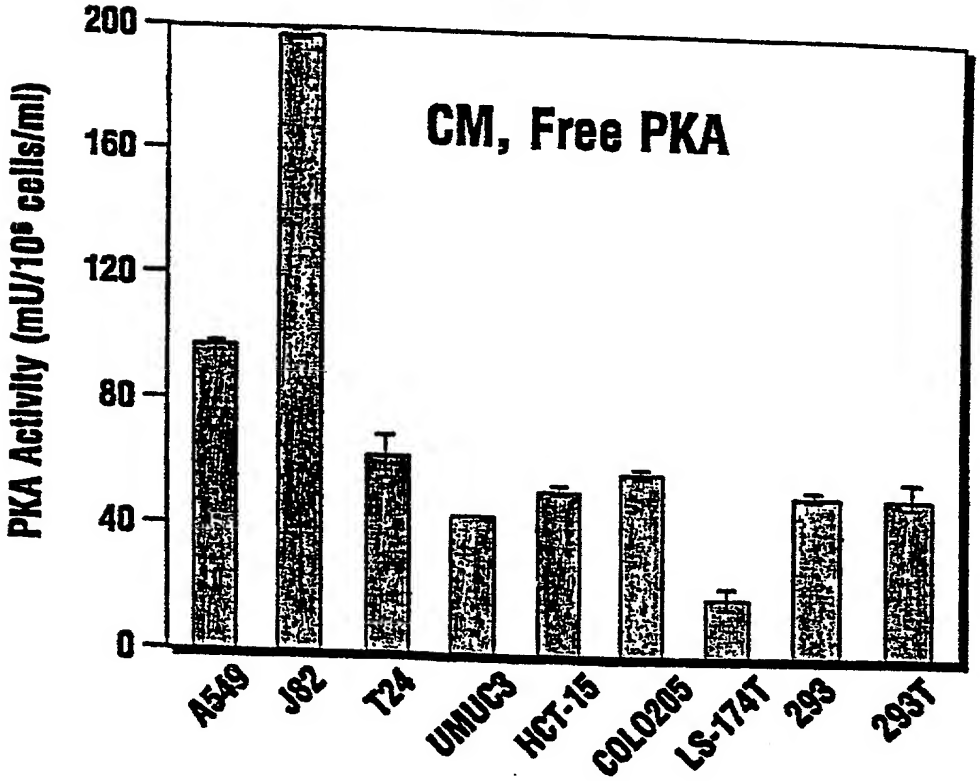
COLO205
LS-174T
293
293T

Fig. 1A


CM, Free PKA
PKA Activity (mU/10⁶ cells/ml)
200
160
120
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HCT-15
COLO205
LS-174T
293
293T

Fig. 1B

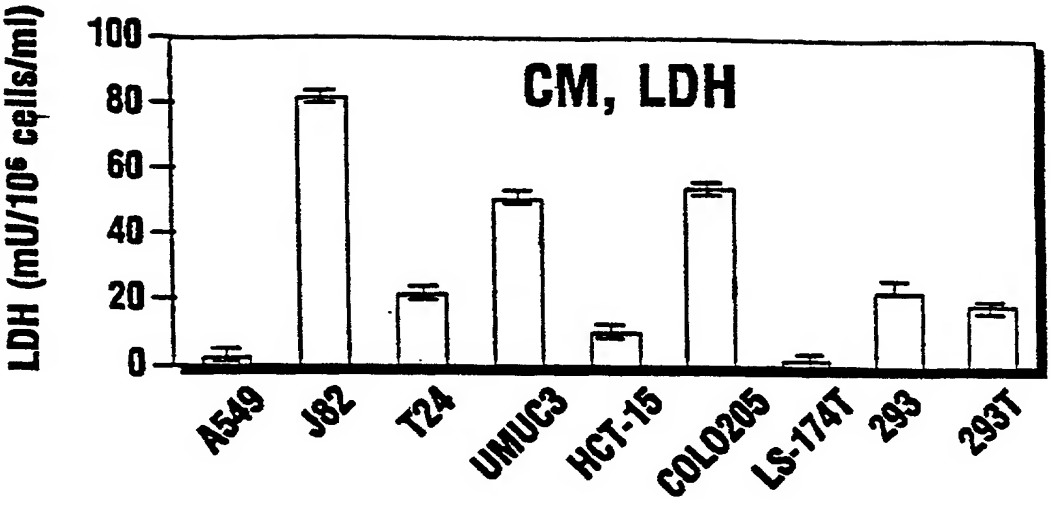
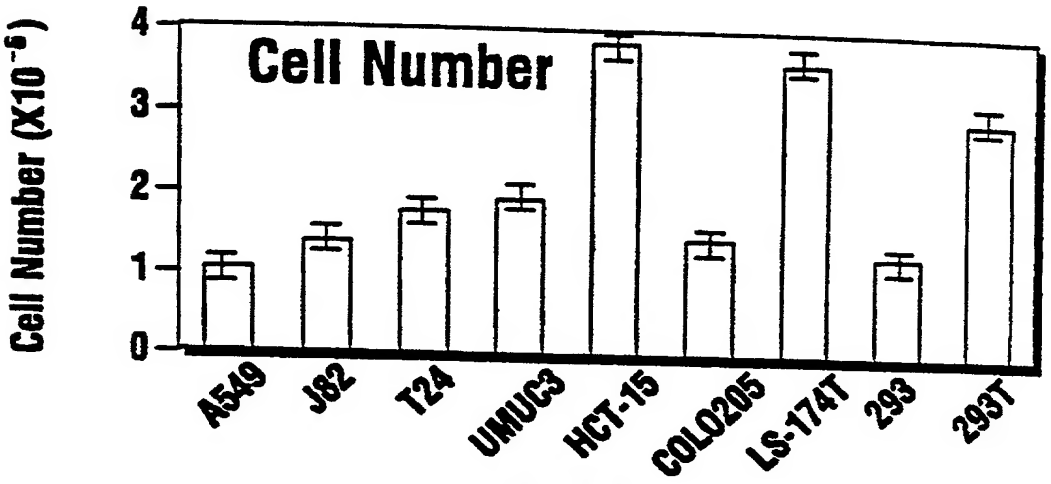


Fig. 1C



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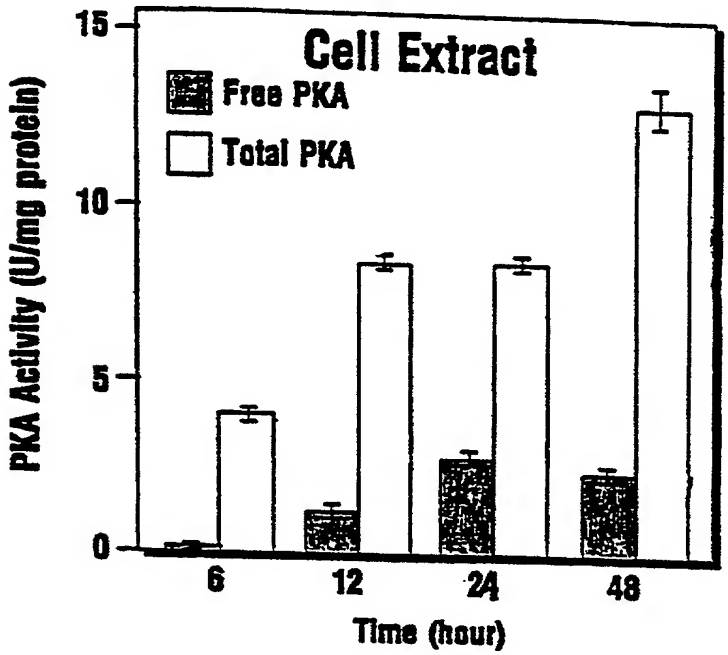


Fig. 1E

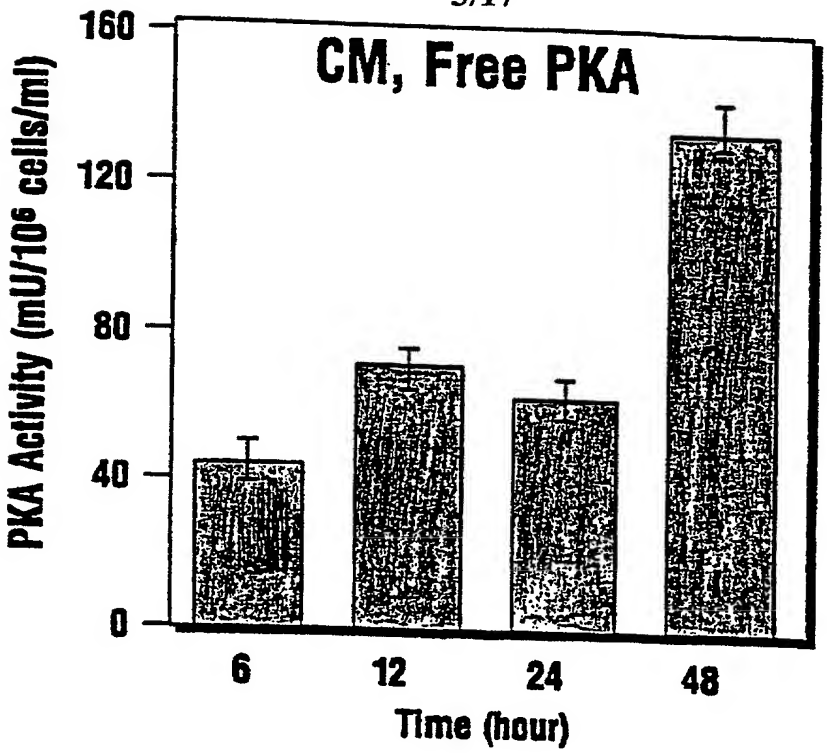


Fig. 1F

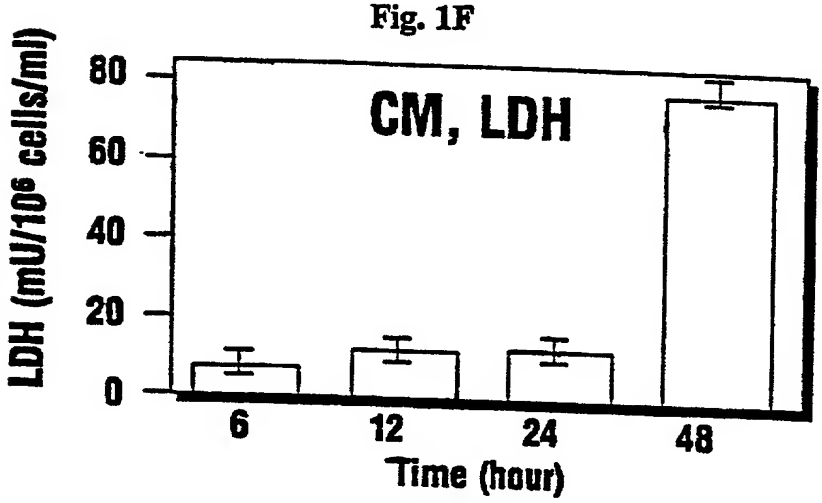


Fig. 1G

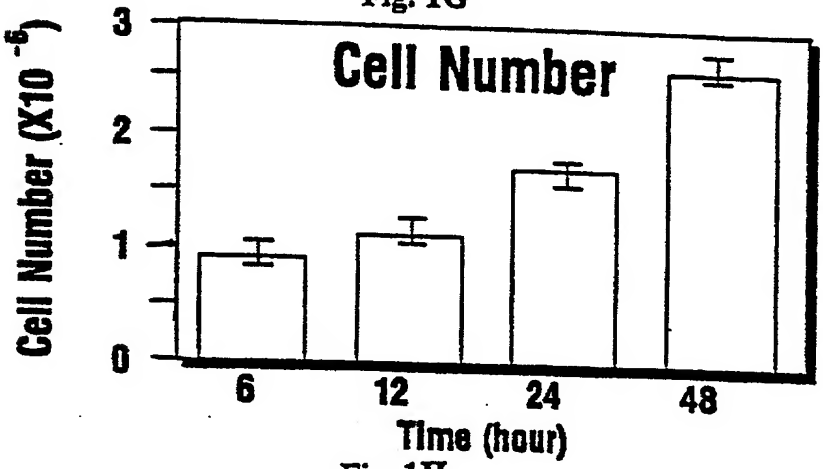


Fig. 1H

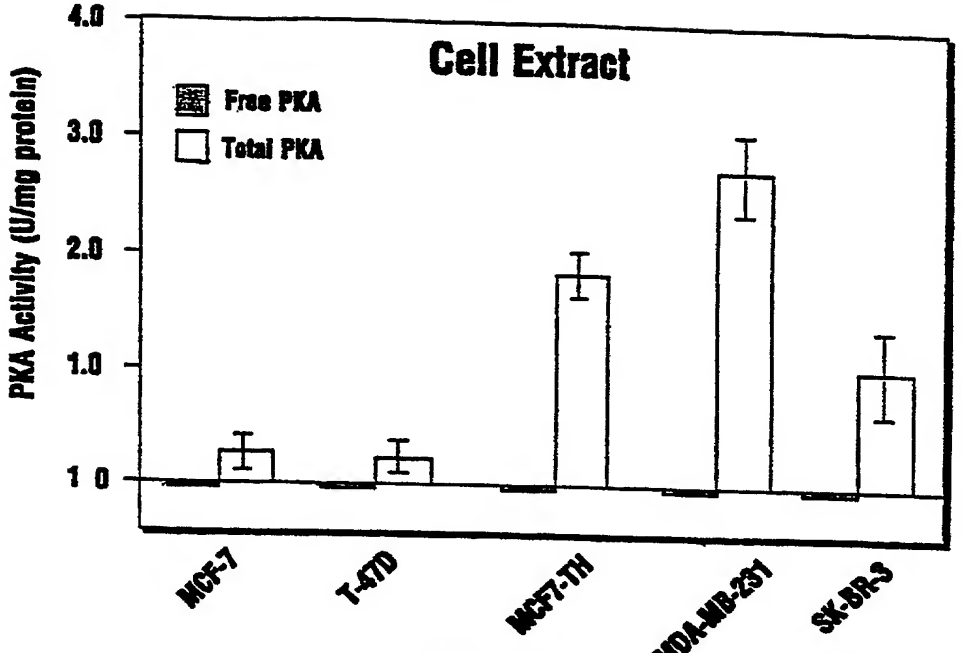


Fig. 2A

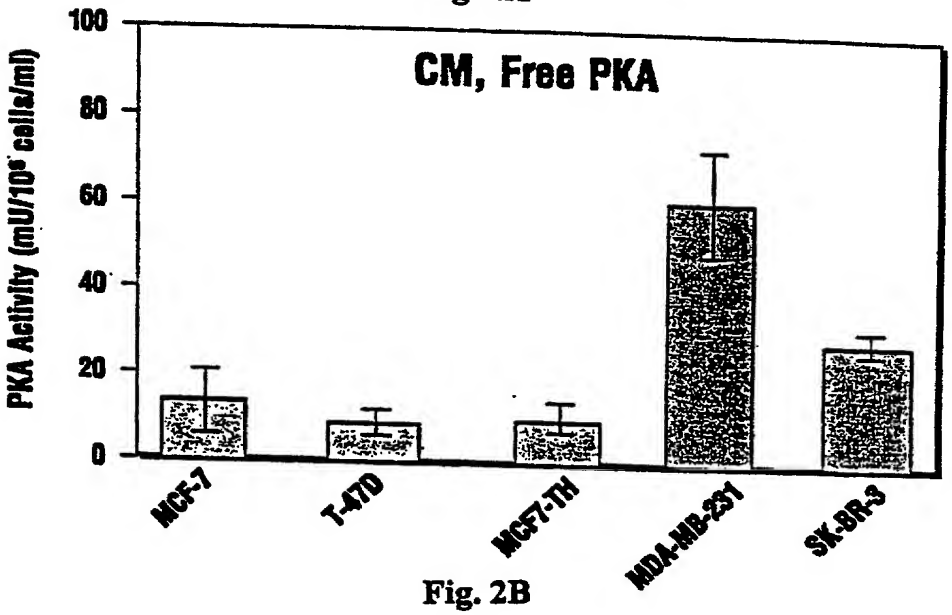


Fig. 2B

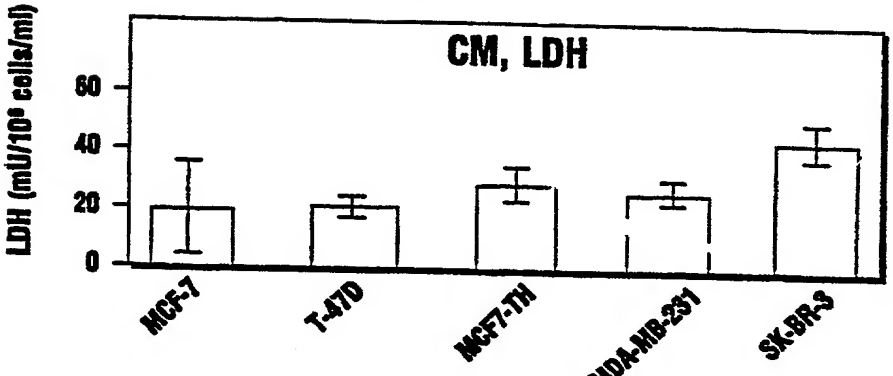


Fig. 2C

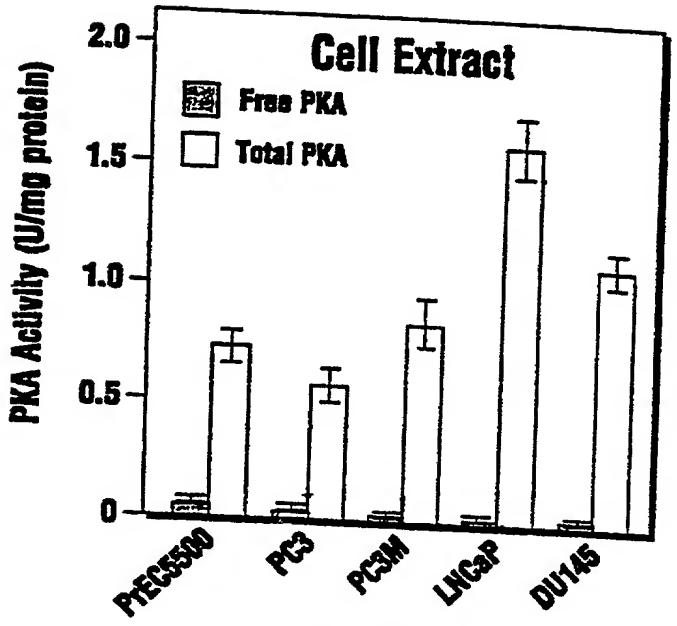


Fig. 2D

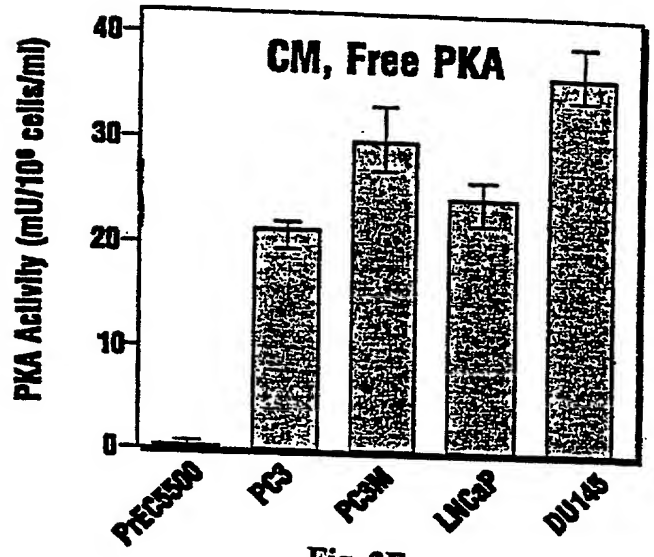


Fig. 2E

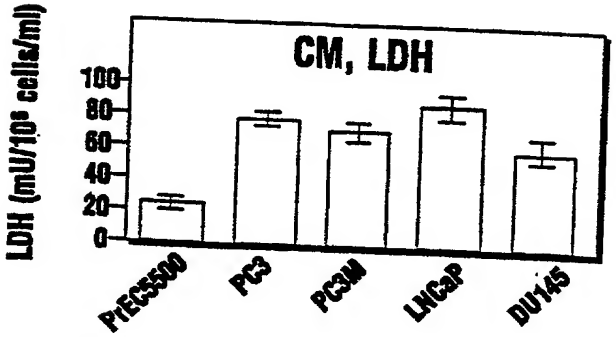


Fig. 2F

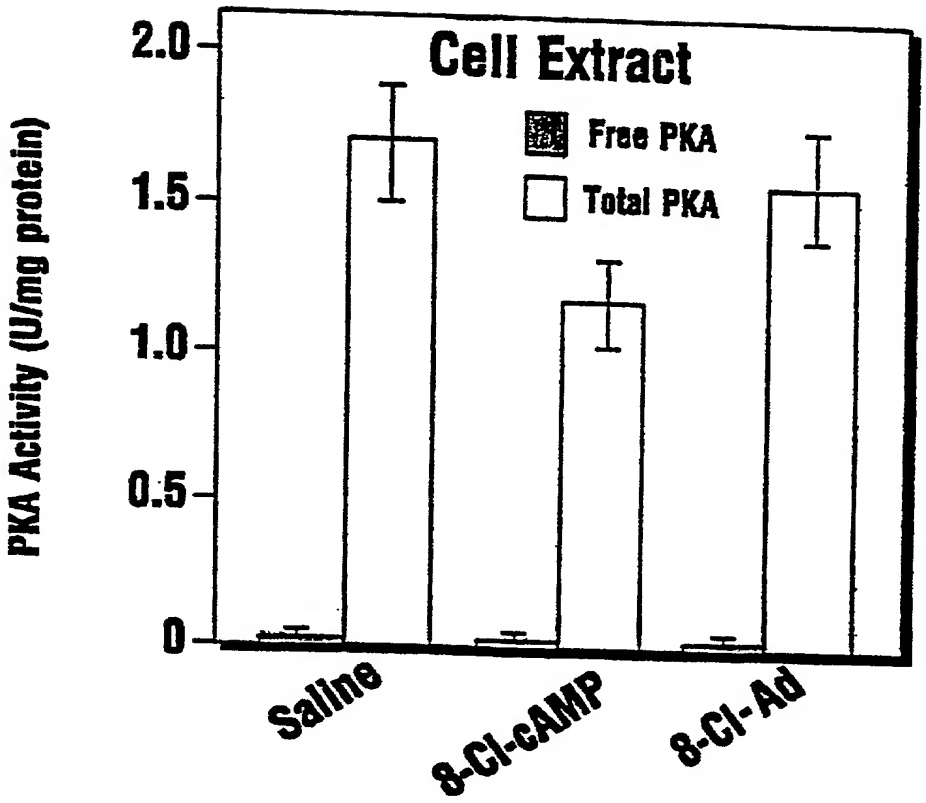

Cell Extract
Free PKA
Total PKA
PKA Activity (U/mg protein)
2.0
1.5
1.0
0.5
0
Saline
8-Cl-cAMP
8-Cl-Ad

Fig. 3A

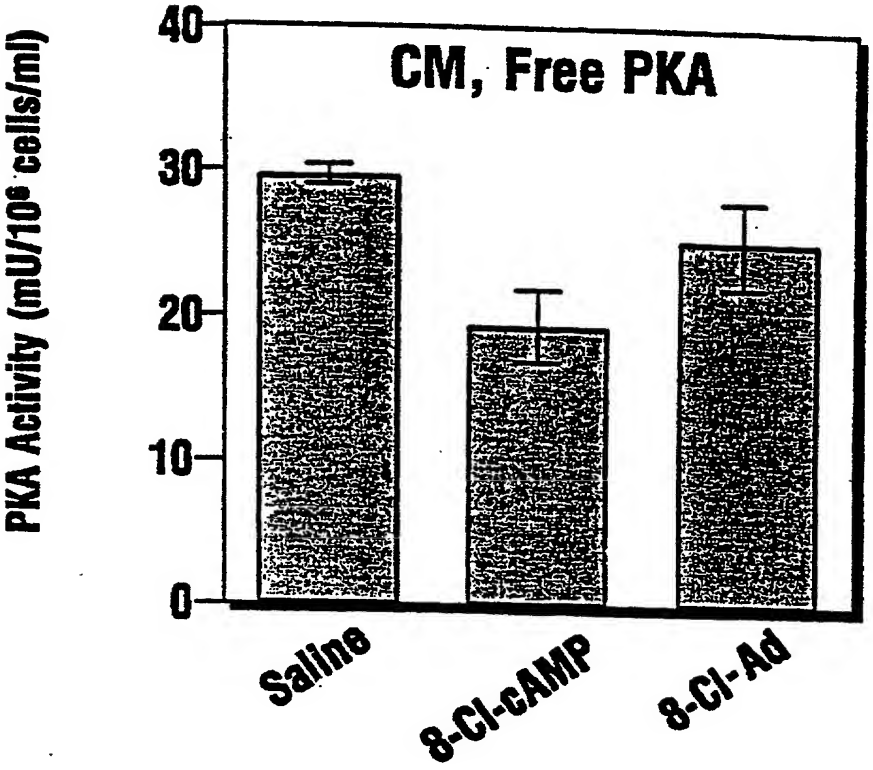

CM, Free PKA
PKA Activity (mU/10⁶ cells/ml)
40
30
20
10
0
Saline
8-Cl-cAMP
8-Cl-Ad

Fig. 3B

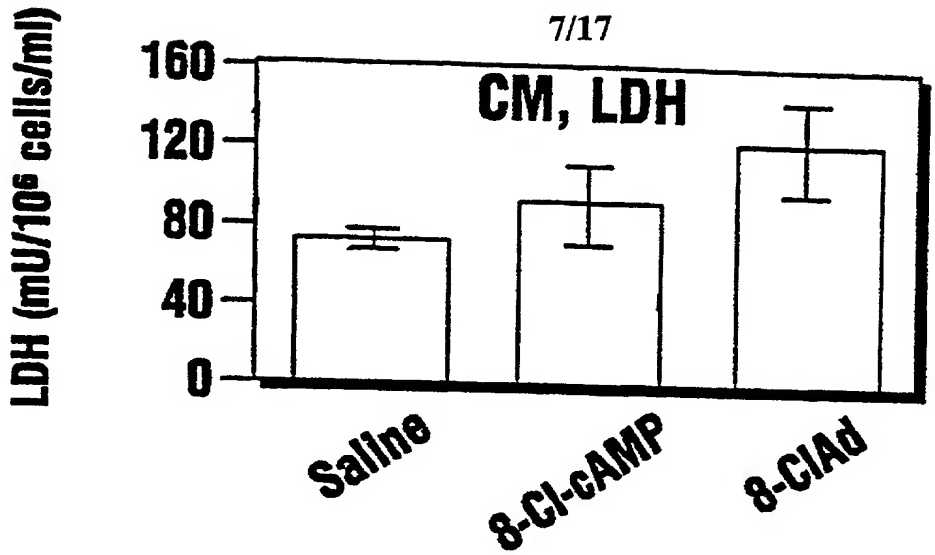

CM, LDH
LDH (mU/10⁶ cells/ml)
160
120
80
40
0
Saline
8-Cl-cAMP
8-ClAd

Fig. 3C

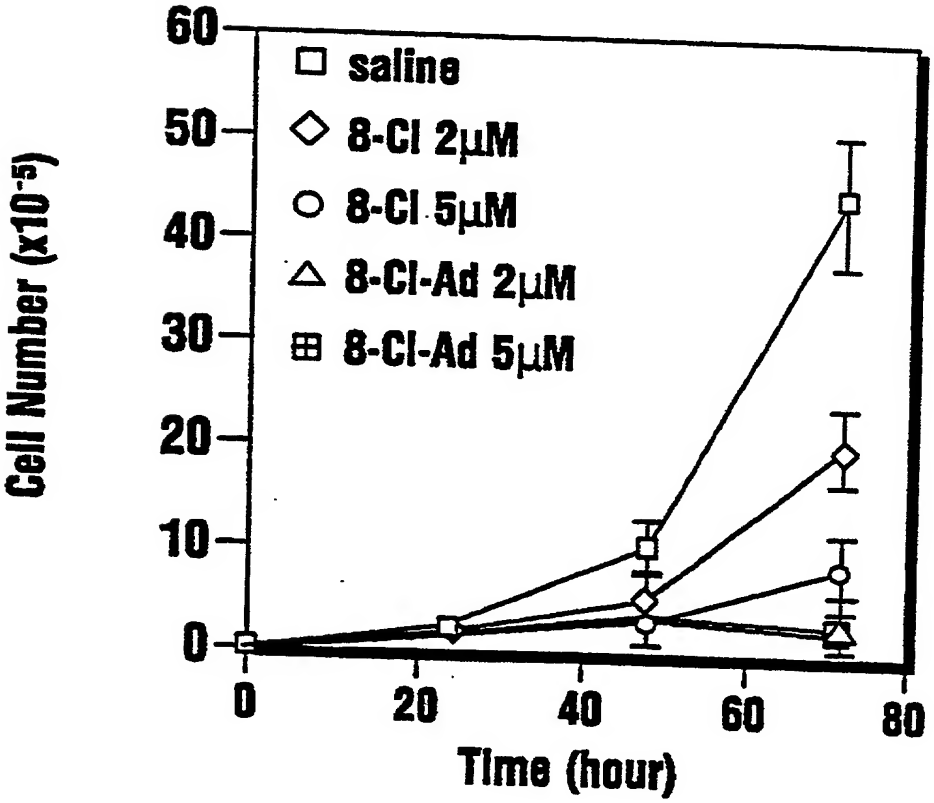

Cell Number (x10⁻⁵)
60
50
40
30
20
10
0
saline
8-Cl 2μM
8-Cl 5μM
8-Cl-Ad 2μM
8-Cl-Ad 5μM
0
20
40
60
80
Time (hour)

Fig. 3D

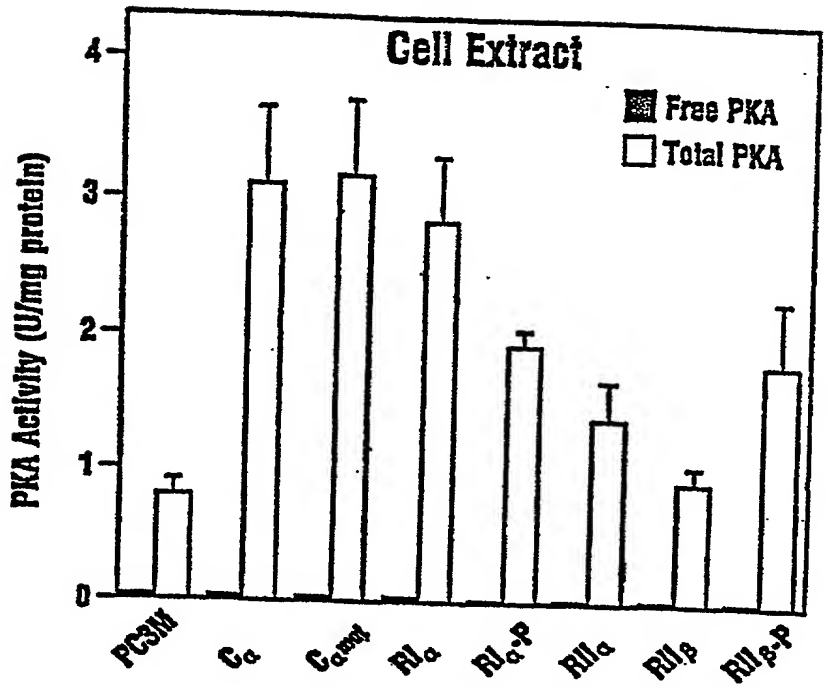


FIG. 3E

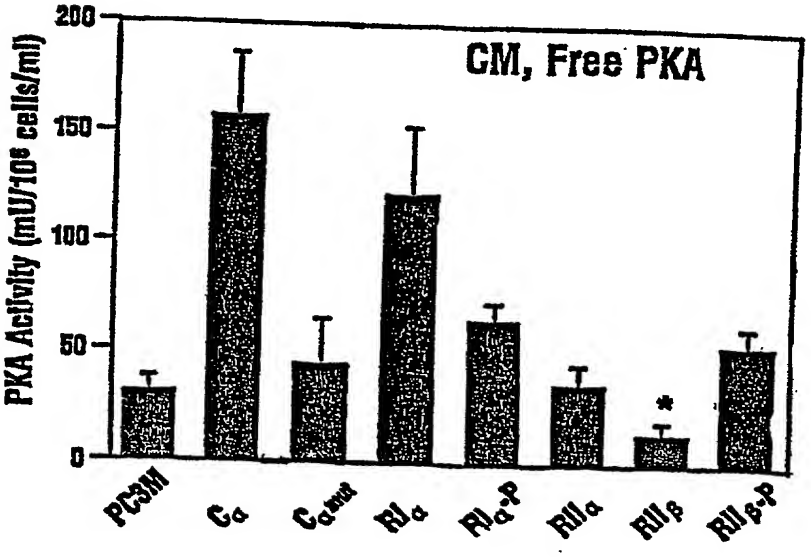


FIG. 3F

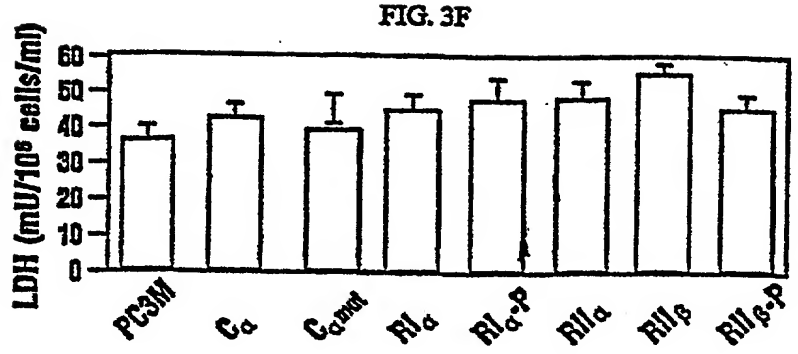


FIG.3G

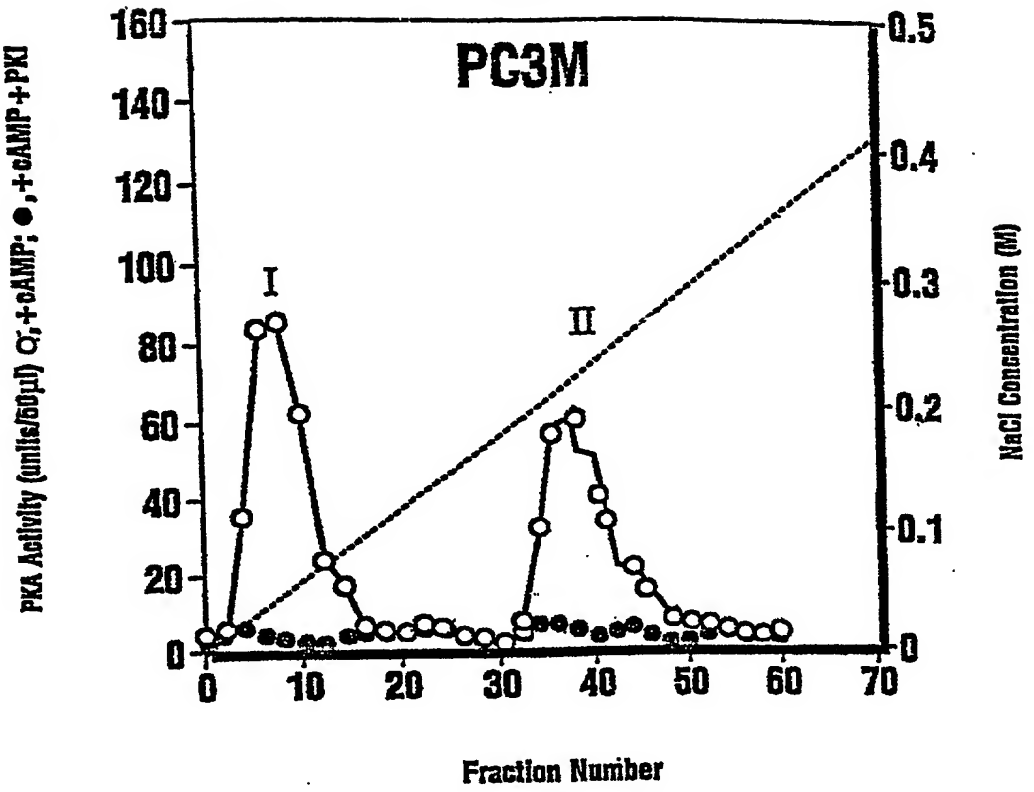

PC3M
PKA Activity (units/60µl) ○,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
I
II
0
20
40
60
80
100
120
140
160
0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70

Fig. 4A

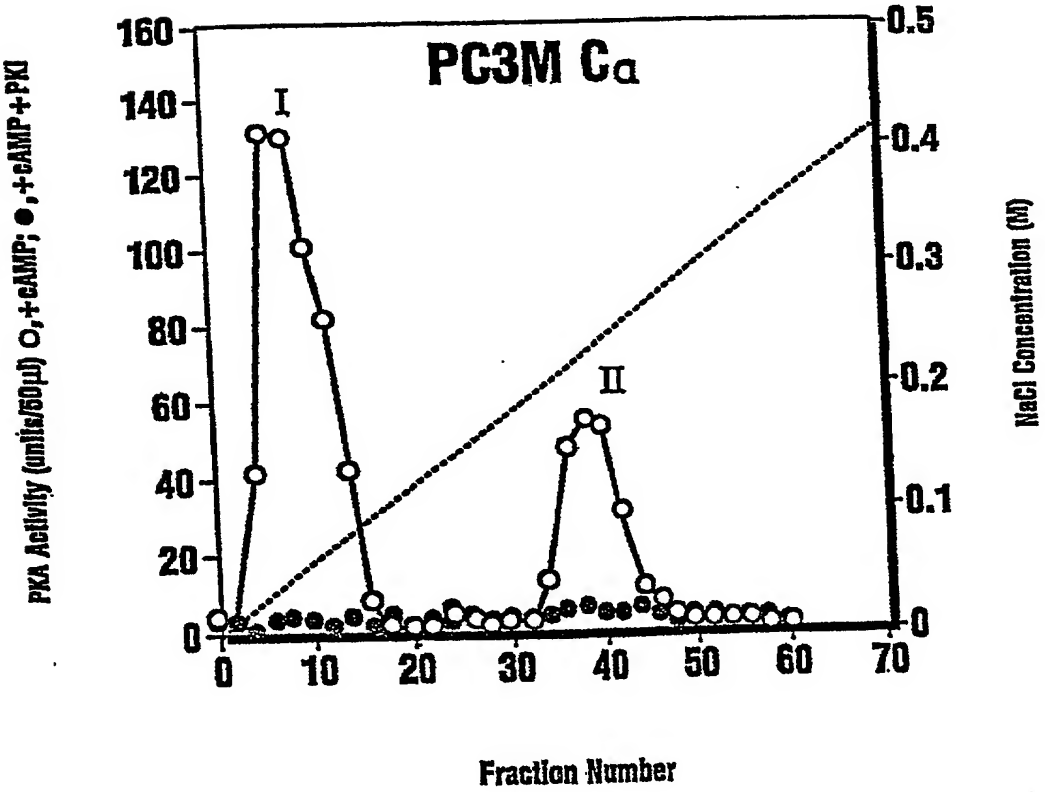

PC3M Cα
PKA Activity (units/60µl) ○,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
I
II
0
20
40
60
80
100
120
140
160
0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70

Fig. 4B

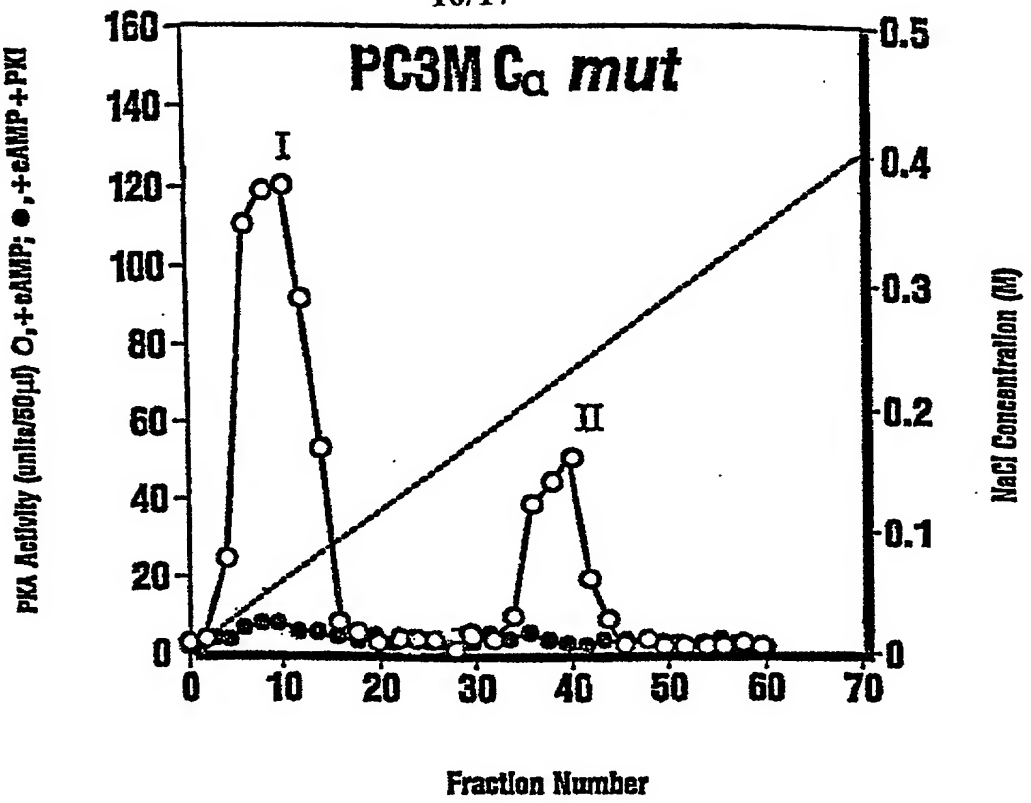

PC3M Cα mut
I
II
PKA Activity (units/50µl) O,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
0
20
40
60
80
100
120
140
160
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70

Fig. 4C

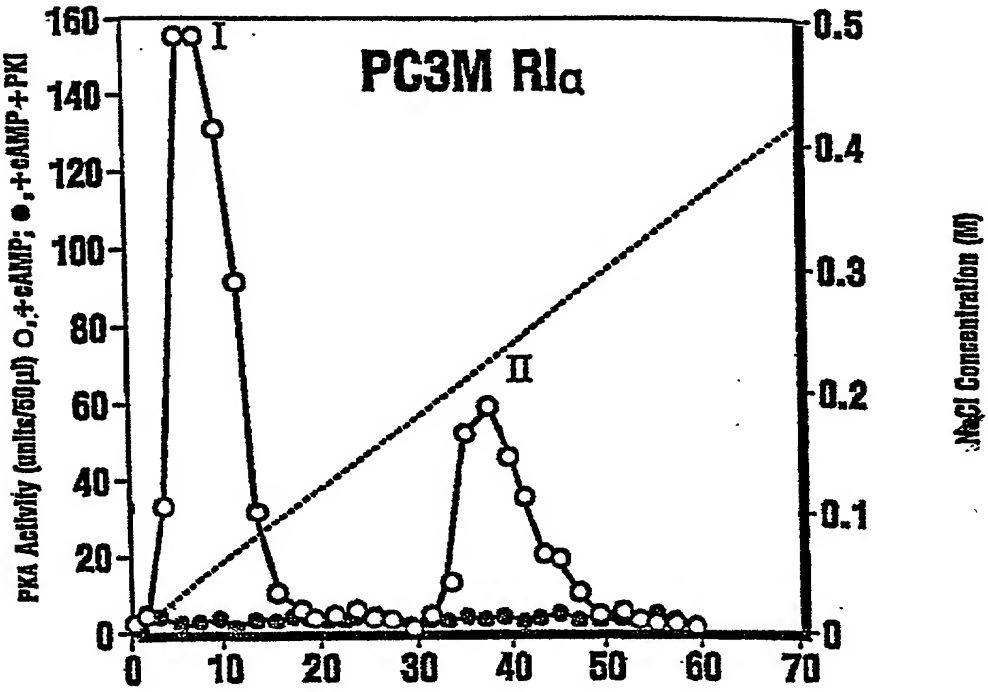

PC3M RIα
I
II
PKA Activity (units/50µl) O,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
0
20
40
60
80
100
120
140
160
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70

Fig. 4D

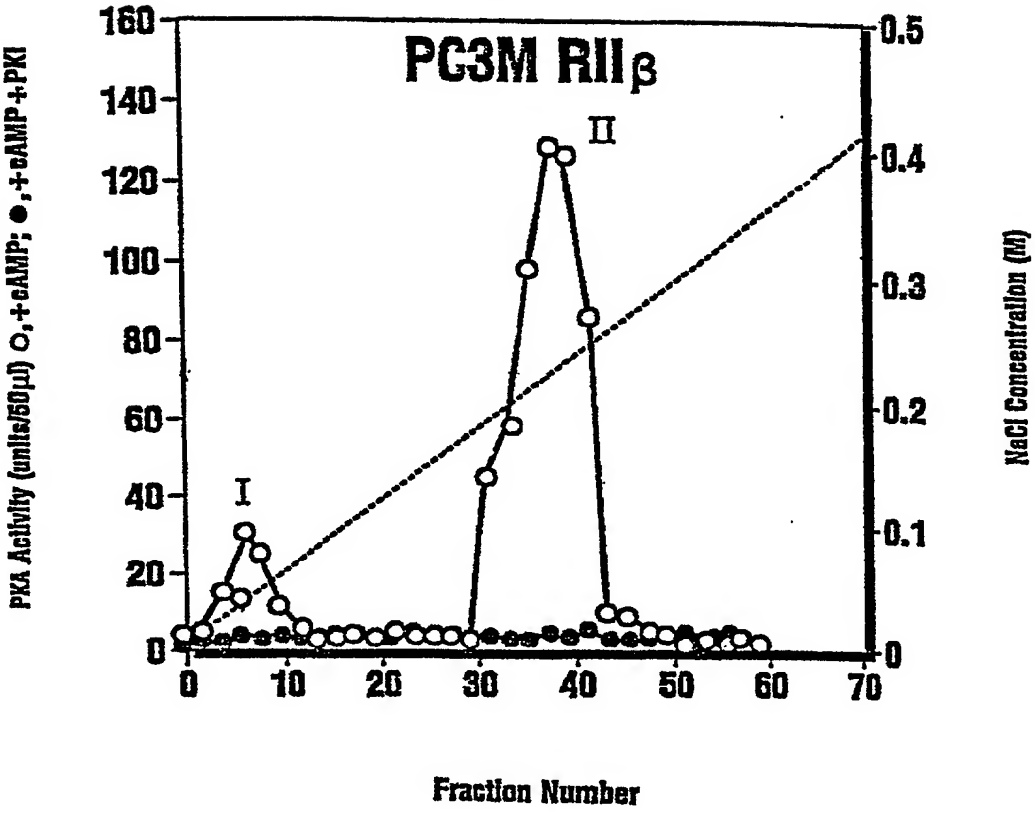

PC3M RIIβ
PKA Activity (units/50µl) O,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
I
II
0
20
40
60
80
100
120
140
160
0.1
0.2
0.3
0.4
0.5
10
30
50
70

Fig. 4E

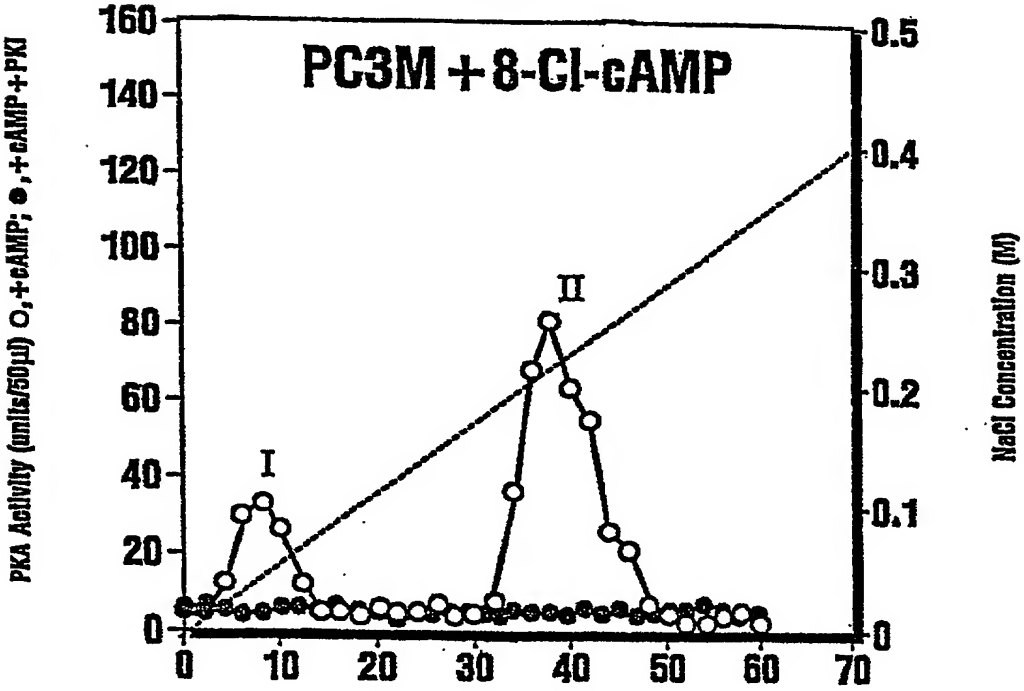

PC3M + 8-Cl-cAMP
PKA Activity (units/50µl) O,+cAMP; ●,+cAMP+PKI
NaCl Concentration (M)
Fraction Number
I
II
0
20
40
60
80
100
120
140
160
0.1
0.2
0.3
0.4
0.5
10
30
50
70

Fig. 4F

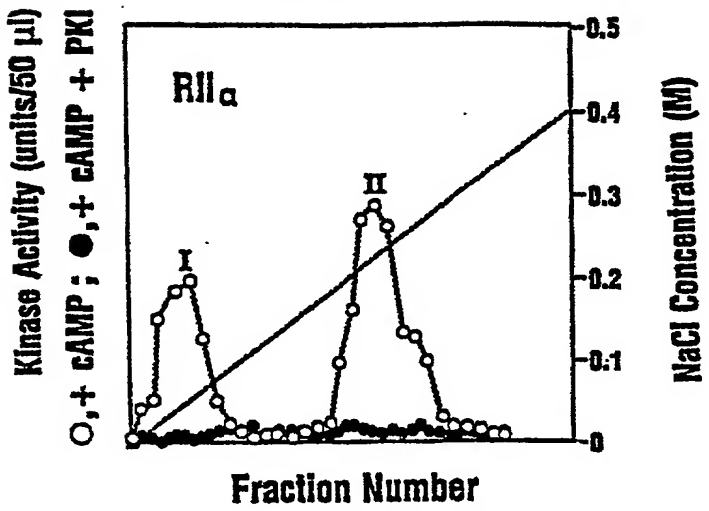


FIG. 4G

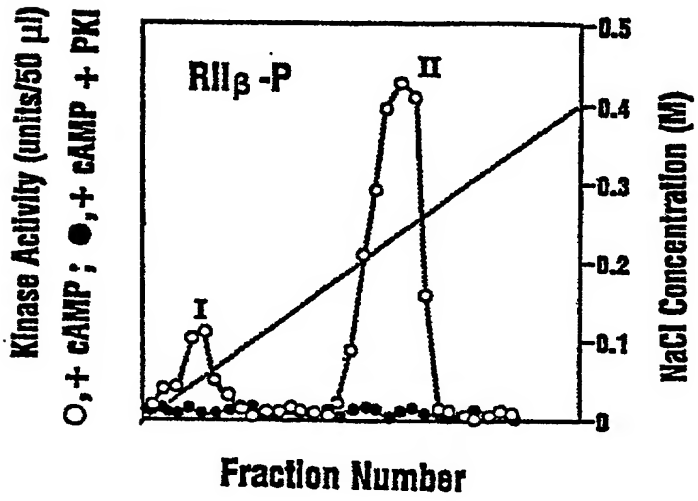


FIG. 4H

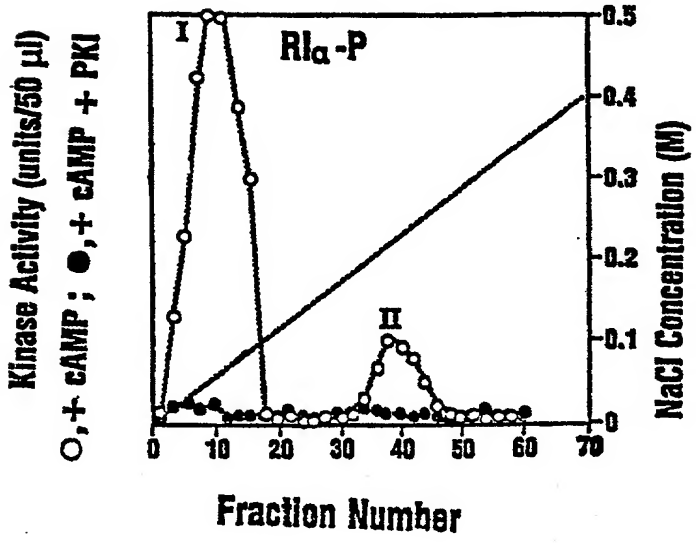


FIG. 4I

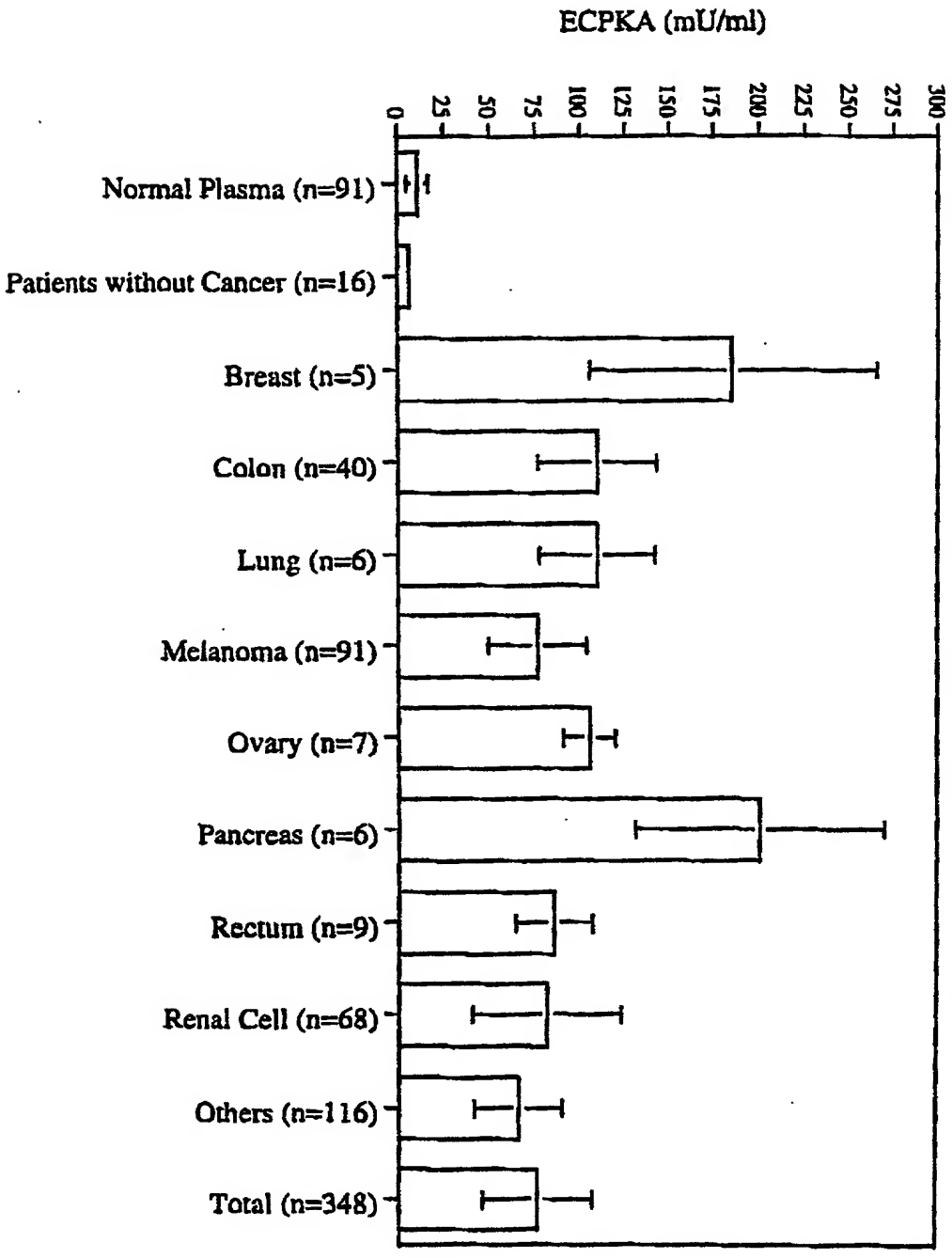
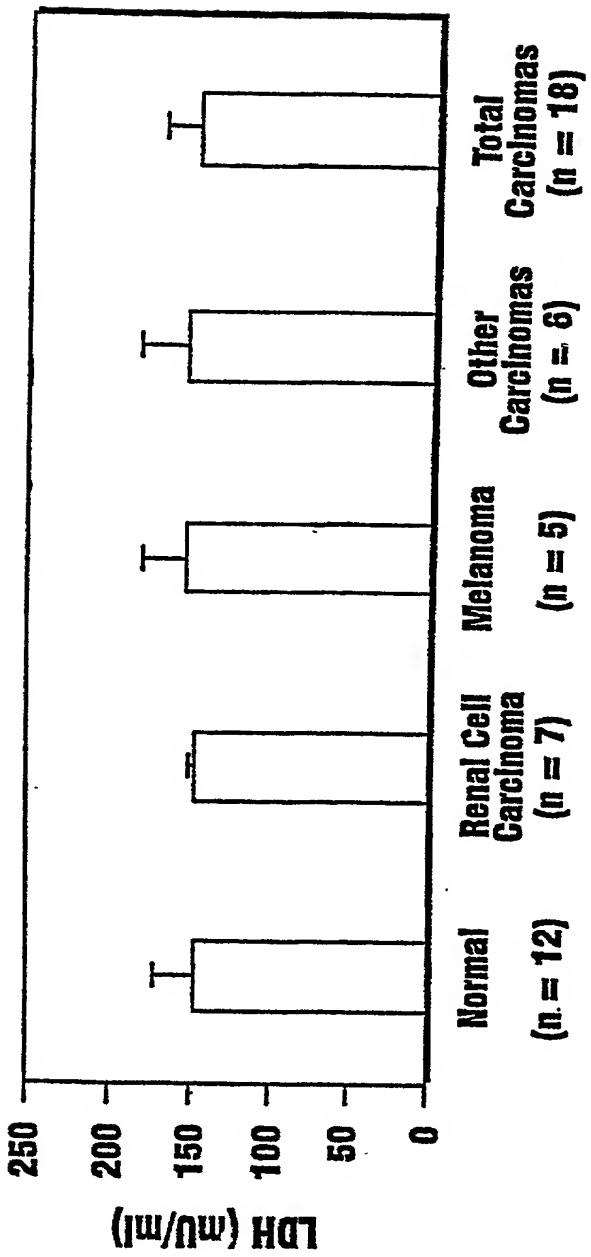

ECPKA (mU/ml)
0
25
50
75
100
125
150
175
200
225
250
275
300
Normal Plasma (n=91)
Patients without Cancer (n=16)
Breast (n=5)
Colon (n=40)
Lung (n=6)
Melanoma (n=91)
Ovary (n=7)
Pancreas (n=6)
Rectum (n=9)
Renal Cell (n=68)
Others (n=116)
Total (n=348)

FIG. 5A

Fig. 5B


LDH (mU/ml)
250
200
150
100
50
0
Normal (n = 12)
Renal Cell Carcinoma (n = 7)
Melanoma (n = 5)
Other Carcinomas (n = 6)
Total Carcinomas (n = 18)

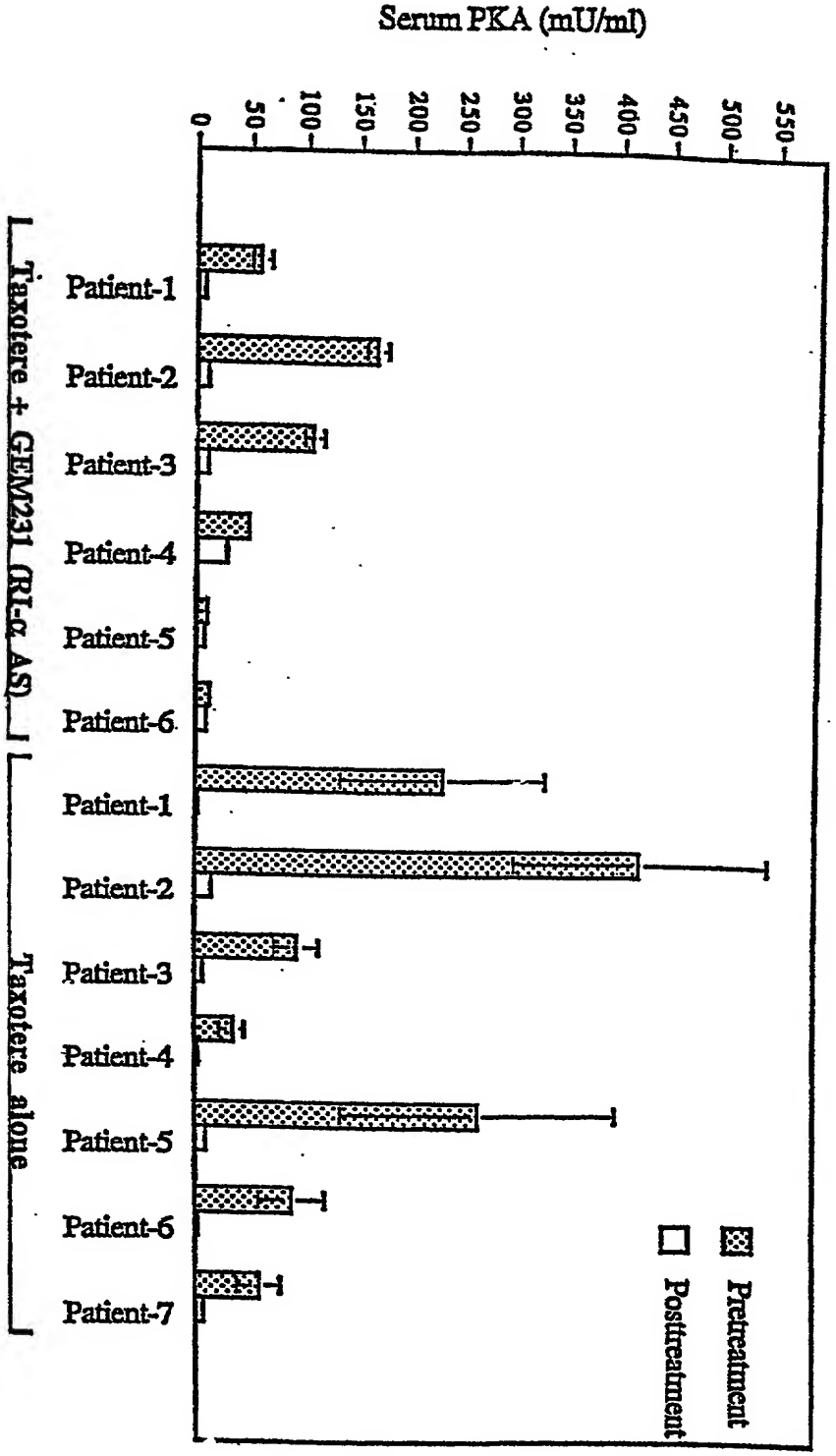

Serum PKA (mU/ml)
0
50
100
150
200
250
300
350
400
450
500
550
Taxotere + GEM231 (RI-α AS)
Taxotere alone
Patient-1
Patient-2
Patient-3
Patient-4
Patient-5
Patient-6
Patient-1
Patient-2
Patient-3
Patient-4
Patient-5
Patient-6
Patient-7
Pretreatment
Posttreatment

FIG. 5C

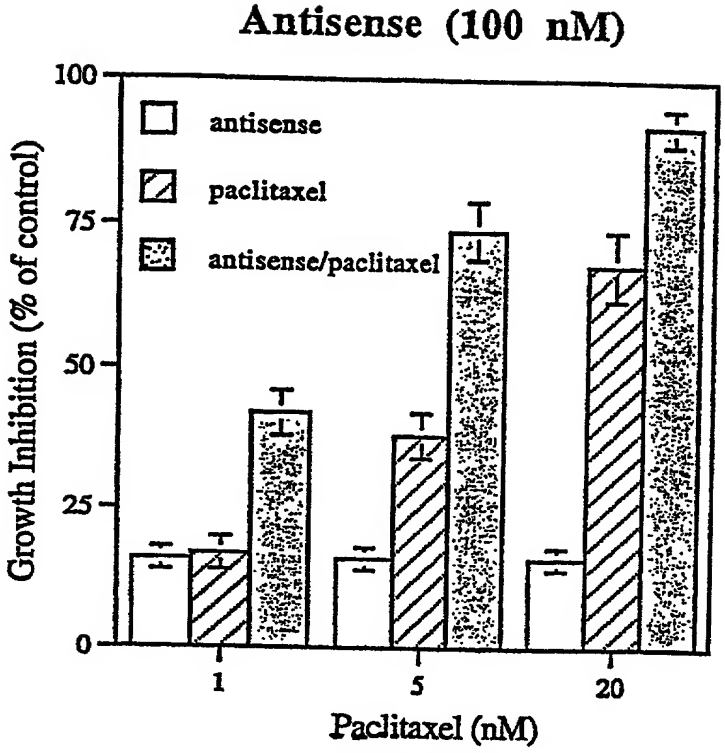

Antisense (100 nM)
100
75
50
25
0
Growth Inhibition (% of control)
antisense
paclitaxel
antisense/paclitaxel
1
5
20
Paclitaxel (nM)

FIG. 6A

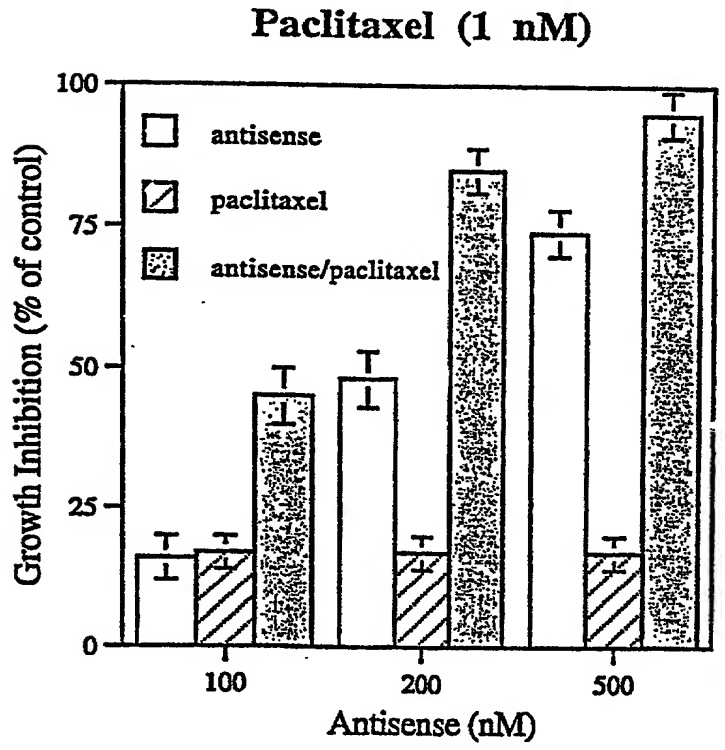

Paclitaxel (1 nM)
100
75
50
25
0
Growth Inhibition (% of control)
antisense
paclitaxel
antisense/paclitaxel
100
200
500
Antisense (nM)

FIG. 6B

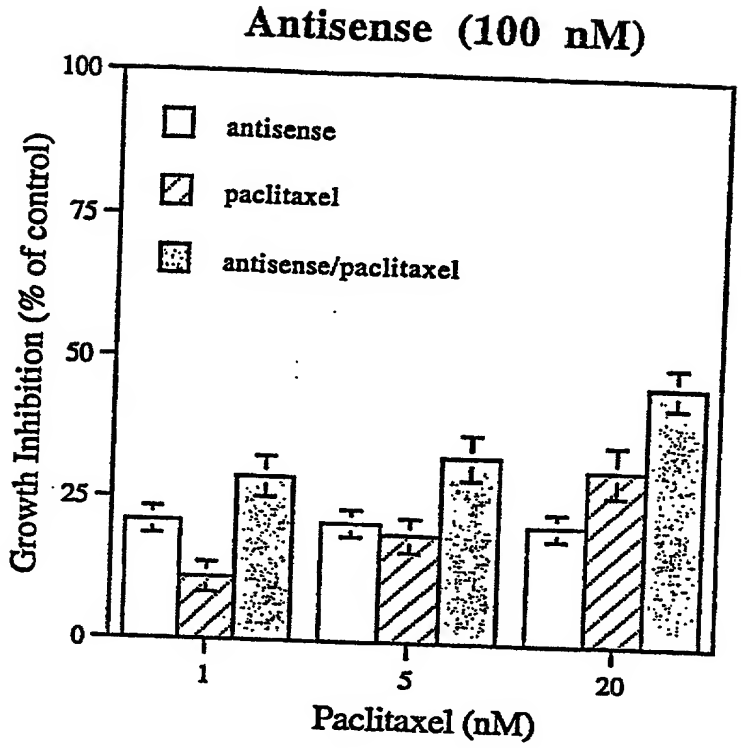

Antisense (100 nM)
100
75
50
25
0
Growth Inhibition (% of control)
antisense
paclitaxel
antisense/paclitaxel
1
5
20
Paclitaxel (nM)

FIG. 6C

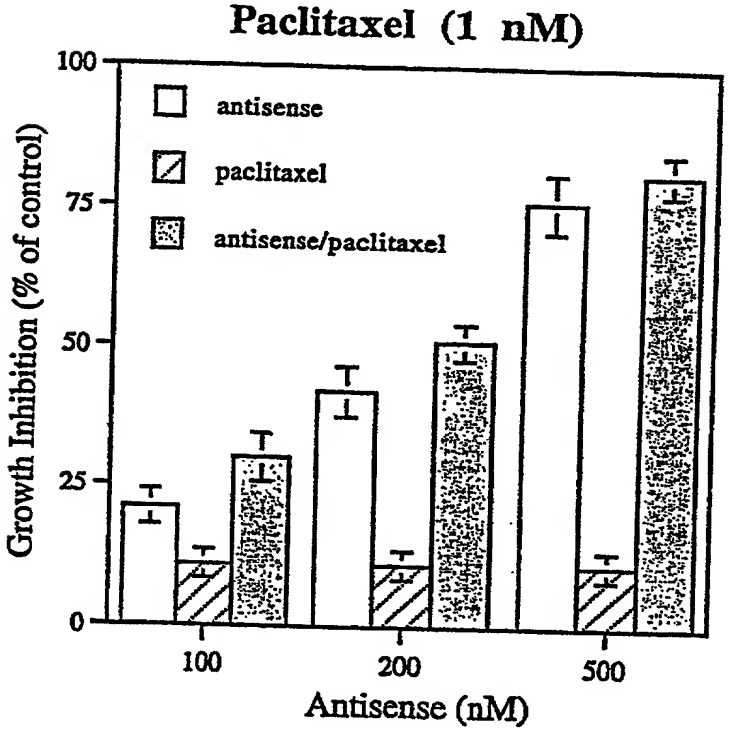

Paclitaxel (1 nM)
100
75
50
25
0
Growth Inhibition (% of control)
antisense
paclitaxel
antisense/paclitaxel
100
200
500
Antisense (nM)

FIG. 6D

PATENT
Attorney Docket No. 214616

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This declaration is of the following type:

☐ original ☐ design ☐ supplemental
☒ national stage of PCT
☐ divisional ☐ continuation ☐ continuation-in-part

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first, and sole inventor *(if only one name is listed below)* or an original, first, and joint inventor *(if plural names are listed below)* of the subject matter which is claimed and for which a patent is sought on the invention entitled:

EXTRACELLULAR cAMP-DEPENDENT PROTEIN KINASE IN DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER

the specification of which:

☐ is attached hereto.
☒ was filed on December 13, 2001 as Application No. 10/018,396 and was amended on *(if applicable)*.
☐ was filed by Express Mail No. *as Application No. not known yet*, and was amended on *(if applicable)*.
☐ was filed on as PCT International Application No. PCT/ and was amended on *(if any)*.

I state that I have reviewed and understand the contents of the specification identified above, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the patentability of the application identified above in accordance with 37 CFR 1.56.

I claim foreign priority benefits under 35 USC 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent, utility model, design registration, or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter and having a filing date before that of the application(s) from which the benefit of priority is claimed.

PRIOR FOREIGN PATENT, UTILITY MODEL, AND DESIGN REGISTRATION APPLICATIONS						
COUNTRY	PRIOR FOREIGN APPLICATION NO.	DATE OF FILING (day,month,year)	PRIORITY CLAIMED			
				YES		NO
				YES		NO
				YES		NO

In re Appln. of Cho-Chung
Attorney Docket No. 214616

I claim the benefit pursuant to 35 USC 119(e) of the following United States provisional patent application(s):

PRIOR U.S. PROVISIONAL PATENT APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC 119(e)	
APPLICATION NO.	DATE OF FILING (day,month,year)
60/140,288	18 June 1999

I claim the benefit pursuant to 35 USC 120 of any United States patent application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this patent application is not disclosed in the prior patent application(s) in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose material information as defined in 37 CFR 1.56 effective between the filing date of the prior patent application(s) and the national or PCT international filing date of this patent application.

PRIOR U.S. PATENT APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC 120					
U.S. PATENT APPLICATIONS			Status *(check one)*		
U.S. APPLICATION NO.	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
1.					
2.					
3.					
PCT APPLICATIONS DESIGNATING THE U.S.			Status *(check one)*		
PCT APPLICATION NO.	PCT FILING DATE (day,month,year)	U.S. APPLICATION NOS. ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
4. PCT/US00/16628	16 June 2000			X	
5.					
6.					

DETAILS OF FOREIGN APPLICATIONS FROM WHICH PRIORITY CLAIMED UNDER 35 USC 119 FOR ABOVE LISTED U.S./PCT APPLICATIONS				
ABOVE APPLICATION. NO.	COUNTRY	APPLICATION NO.	DATE OF FILING (day,month,year)	DATE OF ISSUE (day,month,year)
1.				
2.				
3.				
4.				
5.				
6.				

In re Appln. of Cho-Chung
Attorney Docket No. 214616

As a named inventor, I hereby appoint Leydig, Voit & Mayer, Ltd. to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Customer Number 23460.

I further direct that correspondence concerning this application be directed to Leydig, Voit & Mayer, Ltd.: Customer Number 23460.

I declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1100

Full name of **sole or first inventor:** Yoon S. CHO-CHUNG

Inventor's signature _____

Date 1-15-2002

Country of Citizenship: US

Residence: US
(city/state or country)

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(complete mailing address)